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Discovering bioactive compounds for bone growth
The osteogenicity of marine organism-derived extracts

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Discovering bioactive compounds for bone growth: the osteogenicity of marine organism-derived extracts

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Abstract

Through the current trend for bioprospecting, marine organisms - particularly algae - are becoming increasingly known for their osteogenic potential. Such organisms may provide novel treatment options for osteoporosis and other musculoskeletal conditions, helping to address their large healthcare burden and the limitations of current therapies. This study focused on screening for marine-organism derived osteogenic (promoting bone formation) activity, using extracts produced from a wide range of taxonomic groups sourced from the west coast of Ireland.

Initial work detailed the *in vitro* methods used and their development, such as cell choice and optimisation of key assays included throughout the study. Subsequently, once a robust methodology was established, a large screen of extracts (supplied by the Marine Institute, Galway) was conducted. Initially, this involved testing extracts, produced from a DCM/methanol extraction and reconstituted in DMSO or ethanol, for cytotoxicity, proliferation and differentiation effects on a human foetal osteoblast (hFOB) cell line. Various small scale effects were seen for extracts dissolved in both solvents, along with some pronounced cytotoxicity and significant decreases in proliferation and differentiation. However, neither extract set notably promoted measures of cell activity and thus extracts left over from the original DCM/methanol extraction were also tested. These residues were treated by water or alkaline extraction to produce 'powder extracts', many of which - despite their fairly substantial processing – caused large significant promotions of hFOB proliferation. Interestingly, many of the most promising powder extracts were derived from algal sample material, particularly those from Rhodophyta.

Screening work served to identify a promising concentration range to test with powder extracts, allowing more focused *in vitro* testing using human bone marrow derived stromal cells (hBMSCs). These primary cells give a better indication of cell response within the human body and are therefore more informative than hFOBs used for initial screening work. Powder extracts caused substantial promotions of hBMSC activity measures, including proliferation, differentiation and mineralisation. Taking powder extracts from the epiphytic red algal species *Plocamium lyngbyanum* as an example, increases compared to control of approximately 120% were seen for hBMSC proliferation, +100% for differentiation and +200% for mineralisation. In addition to *in vitro* testing, promising powder extracts were also tested *in vivo* using an operculum area (measuring bone growth) and caudal fin regeneration system. Operculum area system results were highly promising, showing large operculum area

increases with powder extract treatment. With *P. lyngbyanum* , increases of between 50 and 60% were seen over a 3 day growth period, indicating good *in vivo* potential and supporting results seen *in vitro*. Comparatively, caudal fin regeneration system results were less promising, with little significant variation in measures of osteogenesis, regeneration or bone mineral density. This is most likely due to differences between extract preparation and exposure method between the two systems and is something that will be addressed in future work.

Overall, this study details a substantial screening effort for osteogenic activity in marine-organism derived extracts, of which powder extracts showed the most potential. Within this group, red algae were particularly active, showing an excellent ability to promote osteogenic activity *in vitro* and *in vivo*. This may indicate that activity will also be maintained in humans, helping to promote bone growth in those suffering from conditions such as osteoporosis; allowing increases in bone mineral density, a better quality of life and reduced costs for NHS services. Future work should focus on elucidating the exact bioactive component of each extract and its mechanism of effect.

Dissemination of information, publications, funding and awards

Conference presentation- oral

- School of Nursing and Midwifery, Annual Research Showcase (Belfast, September 2017)
- ESB (European Society for Biomaterials), 28th Annual Meeting (Greece, September 2017)
- School of Nursing and Midwifery, Annual Research Showcase (Belfast, September 2016)
- Beaufort Marine Biodiscovery, Annual Meeting (Galway, June 2016)
- NIBES (Northern Ireland Biomedical Engineering Society), Annual Meeting (Belfast, June 2016)
- IOM3 (Institute Materials, Minerals and Mining), Presentation Competition (Belfast, July 2015)

Conference presentation- poster

- BRS (Bone Research Society), Annual Meeting (Bristol, June 2017)
- UKSB (UK Society for Biomaterials), Annual meeting (Belfast, June 2015)
- REMERGE (Regenerative Medicine Research Groups), Conference (Belfast, June 2015)
- NIBES (Northern Ireland Biomedical Engineering Society), Annual Meeting (Belfast, June 2015)
- Postgraduate Centre, Poster Competition (Belfast, May 2015)
- Beaufort Marine Biodiscovery, Annual Meeting (Belfast, December 2015)

Invited speaker

- Surgeons at Royal Victoria hospital – dissemination of research (Belfast, October 2017)
- 2x presentation of research findings for collaborative group (Portugal, Nov 2016 and 2015)

Outreach

- 90-minute outreach presentation, specialist module post-registration nursing students (Belfast, March 2016 and January 2018)
- Science Uncovered public engagement at the Ulster Museum
- Volunteer teaching GCSE English, student subsequently obtained a C grade

Journal publications

1. **Matthew A. Carson**, John Nelson, M. Leonor Cancela, Vincent Laizé, Paulo J. Gavaia, Margaret Rae, Svenja Heesch, Eugene Verzin, Christine Maggs, Brendan Gilmore, Susan A Clarke. *Red algal extracts from Plocamium lyngbyanum and Ceramium secundatum stimulate osteogenic activities in vitro and bone growth in zebrafish.* **Scientific Reports. Published 16th May 2018.**
2. **Matthew A Carson**, John Nelson, M. Leonor Cancela, Vincent Laizé, Paulo J. Gavaia, Margaret Rae, Svenja Heesch, Eugene Verzin, Brendan Gilmore, Susan A Clarke. *Screening for osteogenic activity in extracts Irish marine organisms.* Final stages of preparation. **Target: PLoS ONE.**
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Contents

Abbreviations

ADSCs: adipose tissue-derived stem cells

ALP: alkaline phosphatase

ANOVA: analysis of variance

AP-1: activator protein 1

APC: adenomatous polyposis coli

AR-S: alizarin red-S

BMP: bone morphogenetic proteins

BMP-2: bone morphogenetic protein 2

BMSC: bone marrow stromal cell

BMU: basic multicellular unit

BMU: basic multicellular unit

BP: base pairs

BSP: bone sialoprotein

CFU-fs: colony forming unit-fibroblasts

CITES: convention on international trade in endangered species

CO_2: carbon dioxide

CV: crystal violet

DALYs: disability adjusted life years

DBM: demineralised bone matrix

DCM: dichloromethane

dH_2O: distilled water

DNA: deoxyribonucleic acid

dpf: days post fertilisation

dsDNA: double stranded deoxyribonucleic acid

ECM: extracellular matrix

ERK: extracellular signal-regulated kinases

EtOH: ethanol

FBS: foetal bovine serum

FGF2: fibroblast growth factor 2

g: gram

GC-FID: Gas Chromatography – Flame Ionisation Detector

GFP: green fluorescent protein

GRB2: growth factor receptor bound protein 2

GSK3β: glycogen synthase kinase 3β

h: hour

hBMSC: human bone marrow derived mesenchymal stromal cell

HCl: hydrochloric acid

hFOB: human foetal osteoblast

HPLC: high performance liquid chromatography

ID: identification

IGF: insulin like growth factors

INT: tetrazolium salt

LDH: lactate dehydrogenase

LEF: lymphoid enhancer-binding factor

LMW: low molecular weight

LRP: low density lipoprotein receptor related protein

M: molar

MAPK: mitogen activated protein kinase pathway

M-CSF: macrophage colony-stimulating factor

MeOH: methanol

$MgCl_2$: magnesium chloride

MI: Marine Institute

MPC: mesenchymal progenitor cell

MSC: mesenchymal stem cell; mesenchymal stromal cell; multipotent stromal cell

MW: molecular weight

NaCl: sodium chloride

NAD: nicotinamide adenine dinucleotide

NaOH: sodium hydroxide

NFATc1: nuclear factor of activated T-cells cytoplasmic

NMWL: Nominal Molecular Weight Limit

OPG: osteoprotegerin

PDB: Paget's disease of bone

PBS: phosphate buffered saline

Pen-strep: penicillin-streptomycin

PFA: paraformaldehyde

PMW: postmenopausal women

PTH: parathyroid hormone

QUB: Queen's University Belfast

RANKL: receptor for activation of nuclear factor kappa B ligand

RAY: Mean ray width

REG: total regenerated area

RNA: ribonucleic acid

RPM: revolutions per minute

RTKs: receptor tyrosine kinases

S1P: Sphingosine 1-phosphate

SOS: son of sevenless protein

STU: stump width

TCF: T-cell factor

TGF-β: transforming growth factor *β*

TNF: tumour necrosis factor

TRAFs: TNFR-associated cytoplasmic factors

TRAP: Tartrate-resistant acid phosphatase

Tris-Cl: tris chloride

WHO: World Health Organisation

WSM: water-soluble organic matrix

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Chapter 1

Introduction

1.0 Preface

Bone is a bioceramic composite tissue comprised of a basic mineralized collagen fibril unit (Olszta et al. 2007), and is both formed and maintained through the diverse interactions of a group of cells (Jilka 2003). Bone fulfils many important roles, aiding motility by serving as attachment points for muscle and acting as a mineral store (Harada & Rodan 2003; Huiskes 2000). Clinically, skeletal damage can present a diverse range of treatment challenges, ranging from complex fractures (Stufkens et al. 2011) to pathological bone disorders such as osteoporosis (Alghamdi, van den Beucken & J. a. Jansen 2014), though all centre around the need for stimulation of bone repair (increased osteogenesis). The aim of this project is to identify new osteogenic treatments derived from marine sources. To accomplish this we will screen extracts, derived from marine organisms collected off the west coast of Ireland, for their ability to enhance the proliferation and differentiation of osteogenic precursor cells. These organisms were chosen partly to increase the potential/utilisation of Irish marine resources and partly due to previous indications of osteogenic activity from marine organism (see section 1.3 for more detail).

1.1 Bone structure

1.1.1 Basic macrostructure

Bone tissue is comprised of both organic and inorganic material organised at the macrostructural level into two main types; cortical and cancellous bone. Cortical bone is both hard and dense, forming the outer layer of the tissue as well as the entire shaft of long bones (the diaphysis). Cancellous bone on the other hand is located within this shell, towards the articular end of long bones (epiphysis), and has a honeycomb-like structure made up of interconnecting rods and plates of bone, known as trabeculae (Rho et al. 1998). Cancellous tissue is highly porous, with the intervening space between thin trabeculae filled with either red (containing haematopoietic stem cells) or yellow (adipocytes) bone marrow. Cortical bone also houses bone marrow at its centre, in an area known as the medullary cavity.

At a nanostructural level the organic component of bone is approximately 90% comprised of the fibrous protein collagen type I. Additionally, there are a number of non-collagenous proteins present in the extracellular matrix (ECM) (Ravindran & George 2014; Ingram et al. 1993), which carry out a variety of functions. The non-organic component is comprised of the mineral hydroxyapatite ($Ca_5(PO_4)_3(OH)$), a form of calcium phosphate which appears as

small, thin crystals either in, or upon, collagen fibrils (Weiner & Wagner 1998). This creates the mineralized collagen fibril unit, comprised of three helically wound polypeptide chains with both water and crystals also present within gap regions of the structure (Olszta et al. 2007). Individual fibrils associate with each other to form bundles of fibres, which are then organised into arrays (figure 1.1). Bone is anisotropic, as collagen fibres are laid down in the direction of mechanical loading (Fan et al. 2002), with most healthy adult tissue in the form of lamellar bone. Lamellar bone can be thought of as having a plywood like structure, with parallel fibril arrays arranged in layers of alternating thickness, each of which at a 30^{o} angle to the original (Weiner et al. 1999). As a result, bone generally has excellent mechanical properties, though these vary depending upon the bone in question, between cortical/cancellous bone and with the age of the person (Rho et al. 1998). In general, bones have a high compressive strength and whilst brittle are supported by significant elasticity, helping them bear the load of the body and protect vital organs. Further remodelling occurs in cortical bone to produce structures known as osteons, formed of concentric rings of lamellae around a central, or haversian, channel, which functions as a blood vessel (Olszta et al. 2007; Weiner & Wagner 1998).

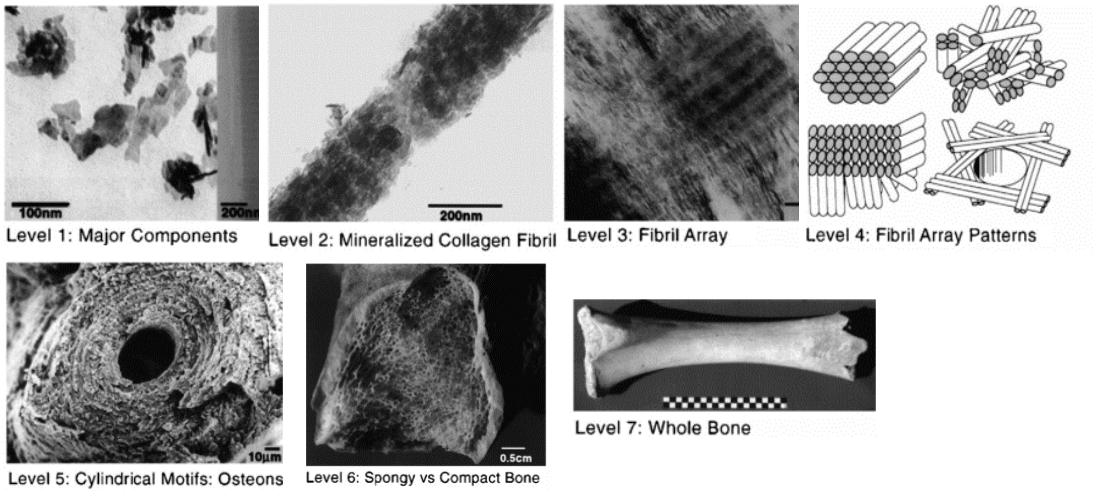


Figure 1.1: adapted figure showing the seven levels of bone structure (Weiner & Wagner 1998).

1.1.2 Bone at the cellular level; remodelling and the basic multicellular unit

Bone is a dynamic tissue that is constantly being broken down and reformed in a process known as bone turnover or remodelling, ensuring the skeleton remains strong and able to cope with its mechanical load (Martin & Seeman 2008). This process is important for mineral homeostasis, particularly calcium for which bone is the only reserve (Heaney 2002); as well as for healing after damage, such as from fractures or microdamage (Lee *et al.* 2002). Bone

remodelling is dependent on a number of different cell types, particularly osteoclasts and osteoblasts. Osteoclasts are multinucleated giant cells which function to break down bone tissue in a process known as resorption (Boyle *et al.* 2003). Alternatively, osteoblasts, with a morphology almost identical to that of fibroblasts, are the cells responsible for bone formation (Ducy *et al.* 2000). Both of these work together to maintain bone structure and to carry out the process of remodelling. A number of other cell types, including bone lining cells, osteocytes, osteomacs and T lymphocytes (see figure 1.3) also aid bone maintenance, functioning together with osteoblasts and osteoclasts in a group known as the basic multicellular unit (BMU) (Kular *et al.* 2012).

1.1.2.1 Osteoclasts

Osteoclasts are located on both periosteal and endosteal surfaces and are formed, in a process known as osteoclastogenesis, through the fusion of mononuclear macrophages or monocytes, derived from hematopoietic stem cells (Boyle et al. 2003). Osteoclastogenesis, and therefore also the degree of resorption, is stimulated by two main chemical factors; macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B ligand (RANKL) (Raggatt & Partridge 2010). The importance of these cytokines in stimulating intracellular signalling pathways was originally demonstrated in mouse model studies. Groups including Kong et al. (1999) and Yoshida et al. (1990) showed that genetic disruption of the *Csf-1* and *Tnfs11* genes (responsible for M-CSF and RANKL production respectively) resulted in osteoclast deficiency and a tendency towards an osteopetrotic (increased density/hardening of bones) phenotype.

As shown in figure 1.2, upon maturation osteoclasts can exist in two functional states: a non-polarised motile state, in which they migrate to the resorption site, and a resorptive state. Resorptive state osteoclasts are polarised and have specific membrane domains, such as an organelle free sealing zone separating the acidic environment

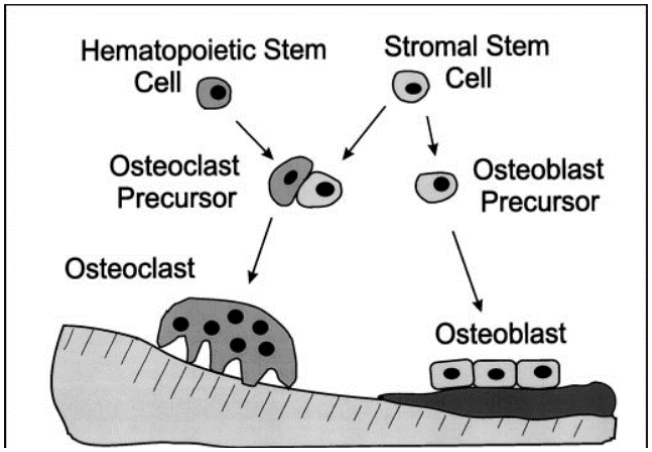


Figure 1.2: a simplified representation of osteoclast and osteoblast development. Stromal stem cells can interact with hematopoietic stem cells to promote osteoclast formation. This figure highlights the morphological differences between precursors and the mature cell types. Figure from (Raisz 1999).

required for resorption from the rest of the cell (Kular et al. 2012). In addition to this compartmentalization there is conspicuous folding of the plasma membrane in the mature cell, creating a ruffled border. This membrane functions to produce a microenvironment between the cell and bone surface and to transport acidifying vesicles and ions (Teitelbaum 2000). These subsequently lower the extracellular pH to 4.5, leading to dissolution of the hydroxyapatitie mineral phase of bone beneath the cell. Consequently, the organic collagen phase of bone is broken down by the secretion of the lysosomal protease cathepsin K (Gowen et al. 1999). This creates a lacunae/resorption pit on the bone surface and is part of the first phase of bone remodelling, termed the initiation phase (Raisz 1999). Figure 1.3 shows the BMU in the process of remodelling for both cortical and cancellous bone. Here, the initiation phase of remodelling involves osteoclast precursor recruitment, from hematopoietic stem cells associated with the bone marrow/blood supply, and their subsequent differentiation to the mature phenotype.

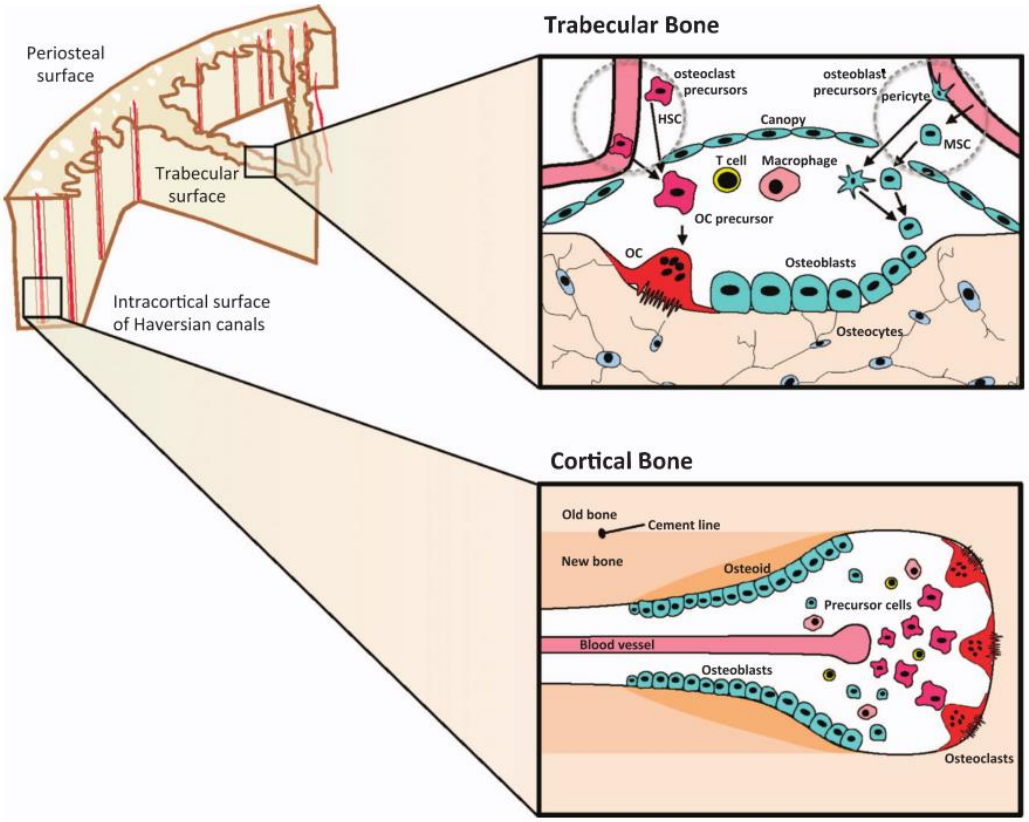


Figure 1.3: the basic multicellular unit, located within both trabecular/cancellous bone as well as cortical bone. Vascularization and bone marrow are key in both cases, supplying hematopoietic precursors which form osteoclasts and MSCs which develop into osteoblasts. These osteoblasts may then form bone lining cells or osteocytes. T cells and macrophages are also derived from blood supply. Figure from (Sims & Martin 2014).

1.1.2.2 Osteoblasts

The second stage of bone remodelling, the reversal phase, is characterised by decreased bone resorption and osteoclast apoptosis, whilst the activity of osteoblasts starts to increase. Osteoblasts are the cells responsible for bone formation and have a morphology almost identical to fibroblasts (Ducy 2000), although mature osteoblasts are cuboidal in shape and have a well-developed Golgi apparatus and rough endoplasmic reticulum. These cells are derived from mesenchymal stem cells (MSC) present within bone marrow, and have a lineage which involves pre-osteoblast commitment and differentiation to the mature osteoblast form, culminating with the formation of an osteocyte, bone lining cell or eventual apoptosis, as represented in figure 1.4 (Neve et al. 2011; Day et al. 2005).

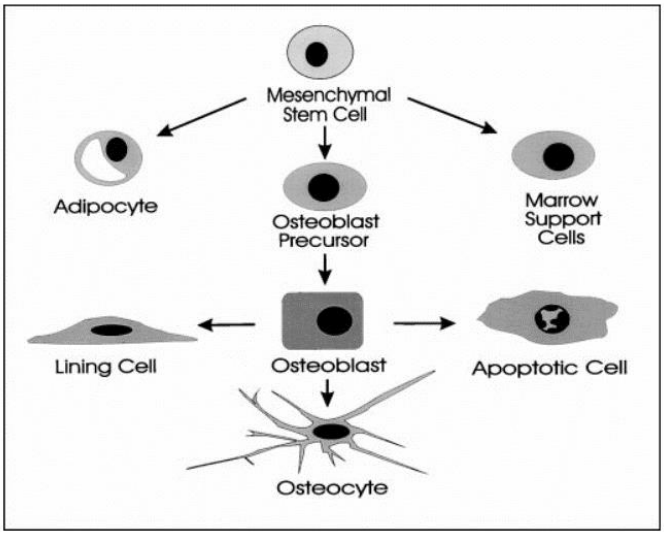


Figure 1.4: the osteogenic lineage from a mesenchymal stem cell (MSC). Included are adipocytes and support cells which MSCs can also form. Also shown are the three potential fates of mature osteoblasts: lining cell/osteocyte formation and cell death. Figure from (Raisz 1999).

Osteoblast differentiation is facilitated by the transcription factor core-binding factor subunit alpha-1 (Cbfa1), also known as runt-related transcription factor 2 (Runx-2). Runx-2 is known as the master regulator of osteoblast differentiation and is one of the only osteoblast specific transcripts (Harada & Rodan 2003; Ducy et al. 1997; Ducy 2000). A number of signalling pathways are known to phosphorylate/activate the Runx-2 transcription factor [see section 1.1.3.1] (Franceschi et al. 2003), the importance of which has also been demonstrated in mice mutation studies. Such work showed that mice deficient in *Cbfa1* have an absence of both osteoblasts and bone, dying shortly after birth (Komori et al. 1997; Otto et al. 1997); whilst forced expression of the transcription factor can induce osteoblast gene expression in non-osteoblastic cells (Ducy et al. 1997).

Once osteoblast precursors are recruited to the area of remodelling and have differentiated to the mature form, the process of bone formation and mineralisation can begin; the termination phase.

The formation of new mineralised bone tissue during remodelling is different to bone formation during foetal development, which occurs by intramembranous or endochondral ossification. Intramembranous ossification involves the direct development of mesenchymal cells into osteoblasts which then form bones including the mandible, clavicle and cranial vault; whilst endochondral ossification involves the transformation of a cartilage template (formed by chondrocytes) into bone, after vascularisation (de Crombrugghe et al. 2001; Ducy 2000). In the remodelling process - after the maximum depth of resorption has been achieved - osteoblasts congregate at the base of the pit. These osteoblasts then begin to secrete an organic matrix, laying down type I collagen fibrils and other non-collagenous proteins to produce a layer of un-mineralised tissue (Kular et al. 2012), known as osteoid. Shortly after the start of collagen deposition, around day 13 of the remodelling process (Globus et al. 1998), mineralisation begins. Though the exact mechanism remains unclear, mineralisation is known to be regulated by osteoblasts predominantly through secreted factors (Matsuo & Irie 2008). For example, crystal formation may be facilitated by 'nucleators' such as bone sialoprotein (BSP), whilst the membrane associated enzyme alkaline phosphatase (ALP) is thought to play a role through regulating phosphorylation of proteins like BSP (Mackie 2003). Both BSP and ALP are markers of osteoblast differentiation (figure 1.5) and are upregulated at the site of bone formation, with collagen synthesis slowly decreasing and being replaced by enhanced mineralisation as the pit becomes filled with osteoid. This eventually results in the formation of new mineralised bone tissue, over a period of around 150 days (Kular *et al.* 2012).

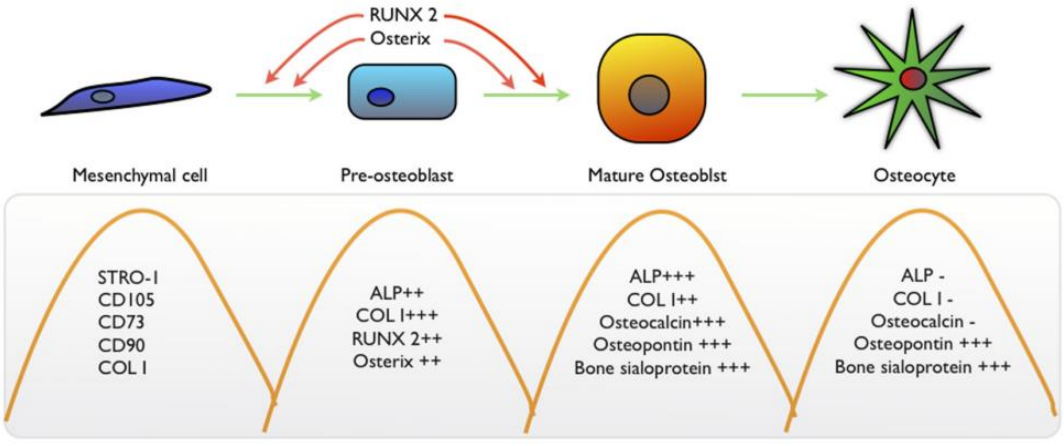


Figure 1.5: sequential changes in cell markers at different stages along the osteoblast lineage, ranging from mesenchymal cells to osteocytes. Figure from (Miron & Zhang 2012).

1.1.2.3 Osteocytes, bone lining cells, osteomacs and T lymphocytes

The remainder of the BMU is made up of cells which support osteoclast and osteoblast function. For example, during the termination phase individual osteoblasts become entombed within the osteoid, to become osteocytes. Osteocytes are a long-lived, terminally differentiated form of osteoblast, which eventually undergo apoptosis (Kular et al. 2012) or are digested during resorption (Hadjidakis & Androulakis 2006). They are believed to act as mechanosensors due to their extensive connections with each other and cells at the bone surface through long filipodial processes, and their ability to influence cellular activity beyond their localised area (Hadjidakis & Androulakis 2006). There is increasing evidence that osteocytes aid the initiation of remodelling, as microdamage-induced apoptosis of these cells correlates with an increase in RANKL (Tatsumi et al. 2007).

Bone lining cells are also terminally differentiated osteoblasts but their role is much less clear. These quiescent cells line most bone surfaces that are not being remodelled, whilst in remodelling areas they relocate to form a canopy (as in figure 1.3), creating a microenvironment which enhances the localization of factors necessary for remodelling (Hauge et al. 2001). Osteomacs - macrophages present on endosteal/periosteal surfaces - form a similar canopy, though on a smaller scale, by covering mature osteoblasts. The function of this may again be to provide factors for the support of osteoblast mineralisation, which they are known to regulate (Chang et al. 2008), or to produce a coupling signal. Finally, T and B lymphocytes are also able to influence the activity of the BMU, particularly that of osteoclasts, as T cells are known to secrete RANKL (Nakashima & Takayanagi 2009).

1.1.2.4 Interactions between cells of the BMU

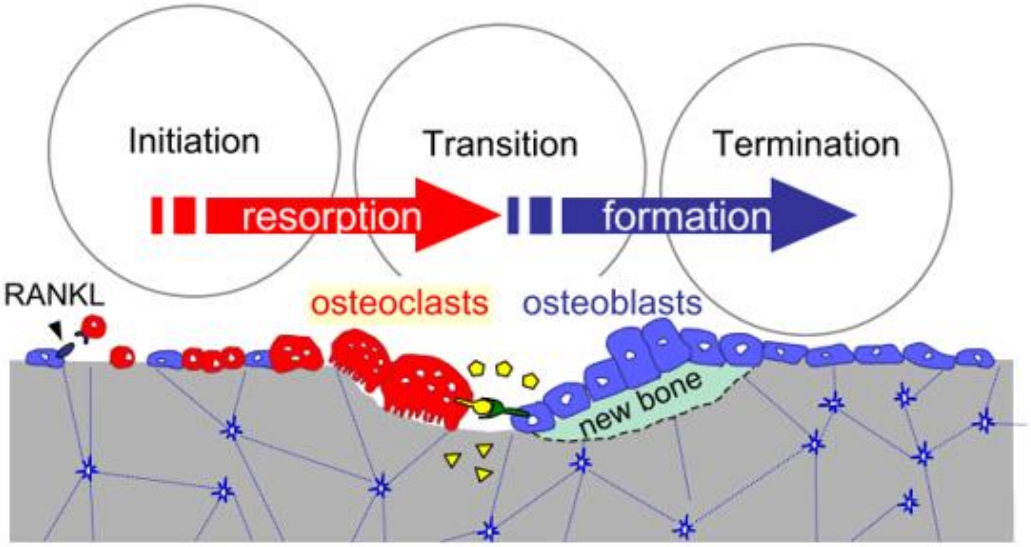


Figure 1.6: representation showing the three phases of bone remodelling: initiation, transition and termination. Osteoclasts (red) resorb bone, stimulated by RANKL (arrow) produced by osteoblasts. Osteoblasts (blue) secret new bone, or osteoid. Regulatory factors can either be liberated from existing bone (triangles), be secreted (hexagons) or be coupling factors needing direct cell to cell interaction (lollipop blue/yellow). Figure from (Matsuo & Irie 2008).

The three stages of bone remodelling, initiation, transition and termination, are represented in figure 1.6. Whilst the cells of the BMU have specific roles in remodelling they are also known to impact the activity of each other, through the production of cytokines, growth factors and other molecules e.g. production of RANKL by osteocytes and T lymphocytes, serving to stimulate osteoclast activity.

A good example of both direct and indirect interactions is the effect of osteoclasts on osteoblast recruitment and function, which serves to stimulate the transition phase of bone remodelling. Changes in topography through lacunae production can stimulate osteoblast activity (Gray et al. 1996), as may recognition of localised factors such as Tartrate-resistant acid phosphatase (TRAP), a marker of osteoclast activity and an important modulator of both collagen degradation and its synthesis by osteoblasts (Roberts et al. 2007). The resorption action of osteoclasts also serves to release factors that are stored in the bone matrix, originally laid down by osteoblasts during bone formation. These factors, for instance bone morphogenetic proteins (BMPs) and insulin like growth factors (IGFs), alter cell signalling to stimulate the differentiation of bone forming cells (Sims & Martin 2014; Kular et al. 2012; Matsuo & Irie 2008). In terms of direct interactions a number of regulatory coupling factors have been identified, for example Sphingosine 1-phosphate (S1P) (Ryu et al. 2006), Ephrin B2 and EphB4 (Martin et al. 2010) and semaphorin 4D (Negishi-Koga et al. 2011). Coupling

involves a membrane bound molecule on one cell binding to a receptor on another, through direct cell to cell contact, bringing about changes in activity. Taking Sphingosine 1-phosphate as an example, this signalling sphingolipid is expressed by osteoclasts and subsequently binds to its receptor on osteoblasts. This serves to increase osteoblast RANKL production and survival, as well as the migration of precursors for both cell types (Sims & Martin 2014; Kular et al. 2012; Ryu et al. 2006).

1.1.3 Major signalling pathways

Direct interactions and regulatory factors produced or liberated by cells, bring about their effects through intracellular signalling pathways. When understanding the response of osteoblasts and osteoclasts to specific factors or bioactives there are a few major signalling pathways that are useful to understand.

1.1.3.1 Osteoblast Wnt-β-catenin pathway

Wnts are a group of highly conserved glycoprotein growth factors that activate cell surface receptors, thus stimulating intracellular signalling pathways controlling a range of cellular processes (Gordon & Nusse 2006). In osteoblasts Wnt operates through the "canonical" pathway, meaning it impacts *β*-catenin levels. In the absence of Wnt, *β*-catenin is held in a destruction complex comprised of the proteins Axin, adenomatous polyposis coli (APC) and the enzyme glycogen synthase kinase 3*β* (GSK3*β*). GSK3*β* phosphorylates *β*-catenin causing its ubiquitination and subsequent proteasomal degradation, keeping cytoplasmic levels of *β*-catenin low. However, if present, Wnt will bind to the co-receptor complex formed of a frizzled protein and the low density lipoprotein receptor related protein (LRP) 5/6. Consequently, Dishevelled protein is activated which inhibits GSK3*β* action; causing an increase in *β*-catenin levels, which ultimately activates lymphoid enhancer-binding factor (LEF) and T-cell factor (TCF) transcription factors (Hoeppner et al. 2009). These cause changes in gene expression that help to promote the early commitment of progenitor cells to the osteoblast lineage, as opposed to chondrocyte development (Day et al. 2005). The importance of the Wnt-*β*-catenin pathway has been demonstrated through mutation/knockout studies, which showed LRP5/6 to be important in maintaining bone mass and strength (Ai et al. 2005; Gong et al. 2001), whilst *β*-catenin influences osteoblast differentiation (Day et al. 2005; Bain et al. 2003).

Major inhibitors of this pathway include the proteins Dickkopf-1 (DKK-1) and sclerostin (Lin et al. 2009), which bind to LRP5/6 meaning Wnt is unable to associate with the receptors. Furthermore, secreted Frizzled-related proteins interact with Wnts directly or bind to Frizzled proteins to inhibit signalling (Hoeppner et al. 2009).

1.1.3.2 Osteoblast - Bone Morphogenetic Proteins

As stated BMPs are one of the best known bioactives used in the treatment of skeletal conditions. These dimeric growth factors are part of the transforming growth factor *β* (TGF*β*) superfamily and have a variety of roles in cellular functioning (Cao & Chen 2005). Specifically in terms of skeletal development, BMP -2, 4, 6, 7 and 9 are known to induce ALP activity in preosteoblastic cell lines, increase osteocalcin levels (another late stage protein marker of differentiation) and enhance mineralisation (Cheng et al. 2003). Moreover, mice respond with increased bone formation after localised calvarial injections of BMP-2 (Chen et al. 1997), whilst transgenic mice with a dominant-negative form of BMP 1-b show reduced postnatal bone formation (Zhao et al. 2002).

The effects of BMPs are transduced through an intracellular signalling pathway, which starts with the binding of the growth factor to serine/threonine kinase receptors, termed type I and type II. Different BMPs bind to the two receptors, which are both necessary for signal transduction as the type II receptor transphosphorylates the type 1 receptor (Miyazono 1999). Subsequently, intracellular signalling is facilitated by Smad proteins, with Smads 1, 5 and 8 activated by the receptor. These can then form a heteromeric complex with Smad 4, which translocates to the nucleus where it activates gene transcription (Miyazono 1999; Kawabata et al. 1998). Regulation of the BMP pathway can be both intracellular; through Smads 6 and 7 which inhibit the pathway by binding to the type I receptor, and extracellular; through Noggin and chordin molecules which form complexes with BMPs (stopping them from binding to the receptors), as shown in figure 1.7 (overleaf).

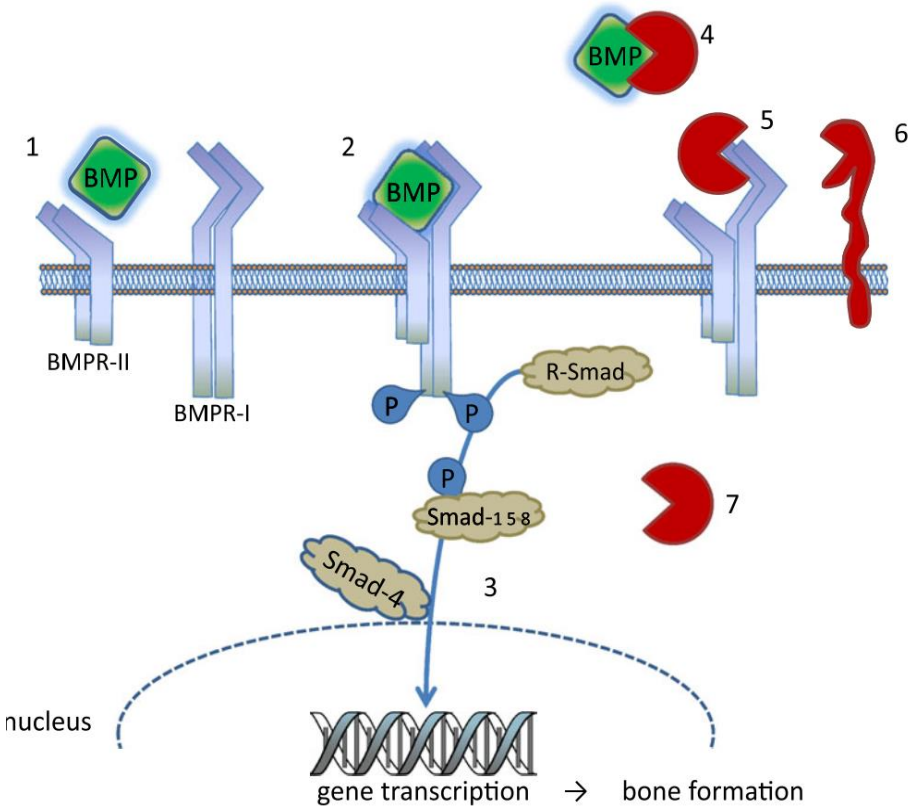


Figure 1.7: 1. BMP binds to receptor II, 2. Receptor II forms a complex with receptor I leading to its phosphorylation. 3. Smads 1, 5 and 8 are then phosphorylated and form a complex with Smad 4 which translocates to the nucleus to influence gene transcription. This signalling pathway is regulated in a number of ways, including 4. Extracellular inhibitors such as noggin and chordin, 5. Receptor inhibitors, 6. Pseudoreceptors and intracellular inhibitors such as 7. Smads 6 and 7. Figure from (Lissenberg Thunnissen et al. 2011)

1.1.3.3 Mitogen Activated Protein Kinase pathway

The mitogen activated protein kinase pathway (MAPK) involves the sequential activation of a series of protein kinases broadly including MAPKKK, MAPKK and MAPK which can be one of four main classes: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase, p-38 MAPKs and ERK5 (Hu et al. 2003). C-Jun N-terminal kinases are involved in stress responses and p38 MAPKs in multiple cellular processes, whilst ERKs make up the classic and best studied pathway influencing proliferation/differentiation.

Various growth factors and cytokines act as activators for the ERK pathway, binding to the extracellular portion of receptor tyrosine kinases (RTKs) which subsequently become phosphorylated. This leads to the binding of growth factor receptor bound protein 2 (GRB2) to the RTK and subsequent binding of son of sevenless protein (SOS) and a GDP binding Ras protein. The latter is catalysed to produce GTP before leaving the complex to bind to the

effector protein B-Raf (Zhang & Liu 2002). B-Raf subsequently activates MEK 1/2 which in turn activates ERK 1/2, a complex which then translocates to the nucleus to influence gene transcription. In osteoblasts the activation of this pathway is dependent on the ECM binding to *β*1 subunit containing integrins on preosteoblasts, or regulated through fibroblast growth factor 2 (FGF2) (Bokui et al. 2008). Ultimately, the action of the ERK pathway in osteoblasts is to accelerate differentiation and aid bone development.

Whilst the ERK class is the best known pathway example, all classes of MAPKs have important roles in regulating gene transcription and cell development. For example, p38 MAPKs are activated along with ERKs during osteoblast differentiation, and when inhibited lead to decreases in mineralisation and ALP activity (Hu et al. 2003). Furthermore, it is not always possible to separate out the actions of the different classes as they often show simultaneous activation, as with ERK 1/2 and JNK 1/2/3 after cytokine stimulation (Bokui et al. 2008). This is also the case for the other osteoblast signalling pathways mentioned in this section, as there is the potential for multiple pathways to be activated at any one time as a result of one or more stimulating factors. For example, multiple signalling pathways are known to converge on the Runx2 transcription factor, including MAPK and BMP pathways (Franceschi et al. 2003), to regulate its expression.

1.1.3.4 Osteoclast

As previously stated osteoclast differentiation is largely facilitated by M-CSF and RANKL. M-CSF cytokines bind to the c-Fms receptor on early osteoclast precursors, facilitating the survival (Teitelbaum 2000) and proliferation of these cells (Neve et al. 2011; Matsumoto et al. 2000). M-CSF is produced by both osteoblasts and stromal cells and, like many of the cytokines discussed so far, operates through multiple pathways (Kular et al. 2012). These include the ERK1/2 pathway and the P13K/Akt pathway (Ross 2006). P13K/Akt serves to degrade or block cell cycle inhibitors such as cyclin D1 and p27/p130, enhancing the proliferation of osteoclast precursors (Ross 2006).

RANKL is a transmembrane protein which is part of the tumour necrosis factor (TNF) superfamily. It is expressed by osteoblasts, T-cells and endothelial cells, and is released in soluble form through proteolysis (Boyle et al. 2003; Teitelbaum 2000). RANKL supports osteoclast differentiation through a variety of different intracellular signalling pathways depending on the TNFR-associated cytoplasmic factors (TRAFs) involved. These pathways include JNK, p38, ERK, Src and the inhibitor of NF-κB kinase (Boyle et al. 2003). Taking the

latter as an example, the adaptor protein TRAF6 is phosphorylated upon the binding of RANKL to RANK, causing a transduction cascade. This cascade leads to the association of IκB kinase (IKK)α, IKK*β* and IKKγ to make the IKK enzyme complex, which subsequently phosphorylates IκB leading to its ubiquitination and protesomal degradation (Otero et al. 2010). IκB normally acts to hold NF-κB in a complex preventing its translocation to the nucleus, though once degraded NF-κB is free to regulate gene expression. The importance of this transcription factor is demonstrated by mutagenesis of its p50/p52 component, which causes osteopetrosis in mice attributed to a lack of mature osteoclasts (Franzoso et al. 1997). Osteopetrosis is also the outcome from mutagenesis of the cFos component of the transcription factor activator protein 1 (AP-1), also stimulated initially by TRAF6 (Grigoriadis et al. 1994).

Whilst p50/p52 subunit proteins and the proto-oncogene cFos are no doubt important for osteoclast development, they are not the sole outcomes of RANKL stimulation. Depending on the pathway in question there are a variety of consequences, including increased expression of genes coding for TRAP and cathepsin K during p38 stimulation (Boyle et al. 2003). One particularly important associated transcription factor is the nuclear factor of activated T-cells cytoplasmic (NFATc1), which is a key osteoclast regulator supporting osteoclastogenesis even in those precursors lacking c-Fos (Matsuo et al. 2004). NF-*κB* and c-Jun are known to interact with NFAT (Zhao et al. 2010), whilst p38 signalling enhances NFATc1 expression downstream of c-Fos (Huang et al. 2006). In addition to this NFATc1 is also upregulated through intracellular calcium signals, via the ITAM pathway which works co-operatively with RANKL (Zhao et al. 2010).

The importance of RANKL in osteoclast development means it is a good target for the endogenous control of bone resorption. This is mainly mediated through osteoprotegerin (OPG), a soluble glycoprotein which acts as a decoy receptor, binding to RANKL and thereby preventing its association with RANK, as in figure 1.8 (Hadjidakis & Androulakis 2006). OPG is mainly produced by osteoblasts and is therefore another example of the coupling between these two cell types. Osteoblasts appear to show sequential changes in the ratio of RANKL to OPG expression, supporting the transition between different phases of bone remodelling (Gori et al. 2000).

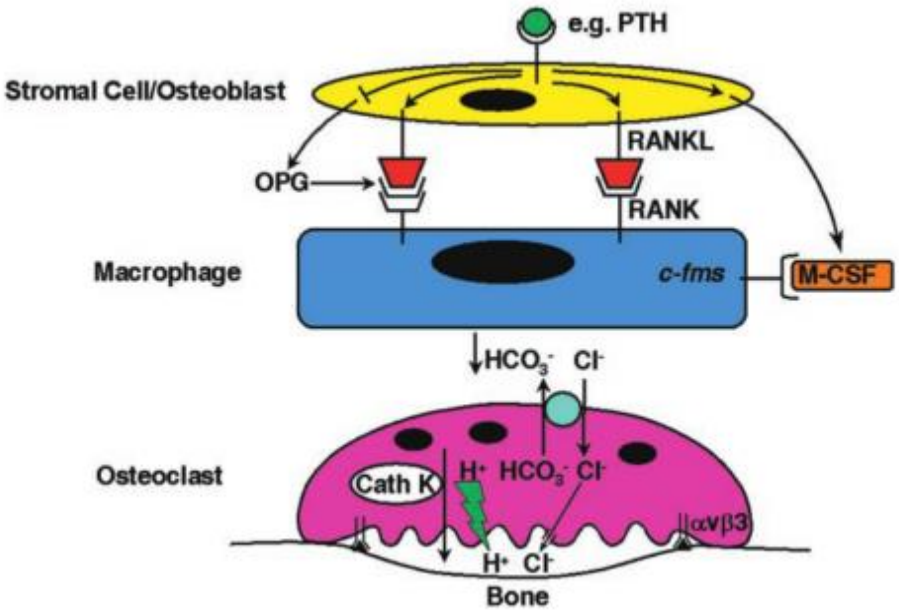


Figure 1.8: the major factors impacting upon osteoclastogenesis. M-CSF and RANKL bind to receptors of macrophages to induce their commitment to the osteoclast phenotype. Each of these factors are secreted by stromal cells/osteoblasts which also produce OPG, which binds to RANKL thus stopping its association with the receptor. PTH can increase expression of RANKL and M-CSF, but can also blunt their expression too. Also shown is the mature osteoclast phenotype polarised on the bone surface in the process of resorption. Figure from (Teitelbaum 2000).

1.2 Clinical Applications

Treatment for bone defects is highly variable, though in general bone regenerative medicine has a triad of key features: a cellular component, a scaffold for tissue growth and the inclusion of bioactive/growth factors (Ringe et al. 2002). Where the bone is significantly damaged or absent a biocompatible scaffold is often required for tissue growth, whilst the importance of the cellular component has already been discussed. On the other hand, bioactive factors are a promising area of study, though are the least developed in terms of clinical usage (Kanakaris et al. 2009). The term 'bioactive' is incredibly broad, referring to a compound or substance which is able to elicit a biological effect. However, in this work the term will be limited to natural products derived from organisms, specifically focusing on marine sources (Gudbjarnason 1999). Bioactives range in structure from small proteins, like the more widely used BMPs (Gomes et al. 2012; Kawabata et al. 1998), to extracellular calcium ions (Nakade et al. 2001). These factors can be present naturally in the body, as with the previous examples, be supplied at increased concentration or be exogenous in their origin (Xiao et al. 2002), the latter being the focus of this study.

Various clinical issues are associated with bone tissue, the most common including fracture repair and pathological conditions such as osteoporosis, osteopetrosis and Paget's disease of bone. In addition to those with specific conditions, the elderly population also constitutes a large treatment group, due to the overall decrease in the structural integrity of bone in later life (Riggs et al. 1998).

1.2.1 Fracture

Trauma can result in bone fractures which tend to heal within 6 to 8 weeks following injury. However, complex fractures, or those presenting in patients with pre-existing conditions like osteoporosis (Charissoux et al. 2013), are much more difficult to treat. Even straightforward fractures may not heal within the expected timeframe, presenting delayed union if healing is not observed after 4 months and non-union after 6 months. The accepted rate of delayed and non-union is between 5-10%, though in long bone fractures these rates are often higher (Tzioupis & Giannoudis 2007). Providing more effective fracture treatment options is therefore necessary, reducing morbidity and mortality for patients and lowering healthcare costs (Charissoux et al. 2013).

Current treatment for situations of mal and non-union involve augments to stimulate bone healing, the gold standard autograft (bone from the patient), allograft (donor bone), demineralised bone matrix (DBM) or synthetic graft material. In graft situations, it is not just a scaffold for growth which is important, but also the inclusion of a cellular population sufficient to promote healing (Gómez-Barrena et al. 2014). The success of autograft compared to other treatments can be attributed to this, as the graft material already contains an immunologically compatible cellular component to support bone formation (Hoffman et al. 2013). However, the collection of bone from patients can cause morbidity at the donor site, increase healing times or may not be suitable for those who are frail (Clarke et al. 2011). It is for these reasons that alternate sources like allograft are used, though healing with this material is often delayed compared to autograft treatment (Hoffman et al. 2013) (Hoffman *et al.* 2013). Similarly, synthetic grafts designed to have high biocompatibility, such as calcium phosphate ceramics, have lower efficacies compared to autograft (Clarke et al. 2011) and poorer mechanical integrity (Rezwan et al. 2006). One way of improving outcomes is to supplement the material with a cellular component. Taking coral grafts as an example, inclusion of bone marrow cells was shown to increase bone surface area compared to treatment with the graft alone (Louisia et al. 1999), though no autograft groups were

included for comparison. In a study that did include autograft groups, ankle fusion rates in the ankle using MSC supplemented allograft (Anderson et al. 2014), were still lower though not to a significant degree.

Bioactives are another option to increase fracture healing and improve the outcomes of graft operations, with the most widely used being that of BMPs. These endogenous growth factors are known to be osteoinductive, stimulating bone formation. Currently BMP-2 and BMP-7 are available for clinical application both in the US (Kanakaris et al. 2009) and UK (Giannoudis & Tzioupis 2005). However, whilst their use is becoming more common, trials are still being conducted to assess their effectiveness. BMP treatment appears to aid healing in clinical trials (Kanakaris et al. 2009; Zimmermann et al. 2009), though the impact and reliability of these and similar studies is often limited by small and/or unequal sample sizes as well as poor experimental design; problems also present in many earlier trials (Garrison et al. 2007). Controversy around the effectiveness of BMPs is enhanced both by their expense (Garrison et al. 2007) and the complex nature of fracture treatment, with outcome success variable between individuals, gender, age groups and the type of bone being treated. However, it appears likely that the use of BMPs will increase with cheaper production methods and optimisation of their application to different fracture types (Lissenberg-Thunnissen et al. 2011). Furthermore, despite the controversy surrounding their effectiveness, the consensus appears to be that BMPs show promise in enhancing bone repair. This highlights the importance of investigating other sources of bioactives in the search for similar and even more effective compounds.

1.2.2 Osteoporosis

As with many pathological bone conditions osteoporosis stems from a breakdown in the regulation of bone remodelling and therefore unbalanced functioning of the BMU. The World Health Organisation (WHO) defines osteoporosis as a bone mineral density 2.5 or more standard deviations below the average value of the young (female) reference (Kanis et al. 1994). Based on the WHOs definition it is estimated that 30% of postmenopausal women (PMW) suffer from the disease, a percentage which increases with age (Kanis et al. 1994). Osteoporosis is characterised by a decrease in bone mass attributed to increased bone resorption by osteoclasts and reduced bone formation by osteoblasts. This decrease in bone mass is accompanied by degradation of the bone microarchitecture and increased fracture risk (Cummins et al. 2011). Osteoporosis tends to impact people in their fourth or fifth decade

of life; particularly PMW, a trait strongly associated with decreasing estrogen levels (Riggs et al. 1998; Albright et al. 1941). This is explained at the cellular level through estrogen receptor alpha signalling, which increases osteoclast activity and causes shorter osteoblast lifespans (Alghamdi, van den Beucken & J. A. Jansen 2014). Additionally, osteoporosis effects bone healing, not only through disrupting the regulation of growth factors and cytokines (Pacifici 1996) but also through impacting MSCs, which show a poorer growth rate in PMW (Marco et al. 2005).

As with any large scale disease estimations, suggestions on affected populations and associated costs vary between studies and location. However, one recent and comprehensive systematic review aimed to assess the global burden attributed to low BMD, and found that that the number of deaths and disability adjusted life years (DALYs) increased from 1990 to 2010. This represents an almost doubling of the total global burden from 0.12 to 0.21% (Sànchez-Riera et al. 2014). Furthermore, a recent report estimated that the economic burden of osteoporosis was €37.4 billion for Europe (27 countries) as a whole in 2010, with the UK making up a significant €5.4 billion of this total (Hernlund et al. 2013). This is particularly daunting considering the prevalence of the condition in aged populations, coupled with estimations that by 2030 20% of Europeans and 30% of the US population will be over 65 (Christensen et al. 2009). It is therefore in the best interests of patients, governments and health services to develop the most effective treatments possible. Currently available therapies are numerous, ranging from physical exercise programmes to pharmacological treatments (Khajuria et al. 2011). However, all current treatments present implementation and efficacy issues, for instance the cost and numerous side effects associated with hormone therapy (Khajuria et al. 2011). It would therefore be beneficial to develop an effective bioactive to aid bone healing and/or manage bone maintenance in later life; allowing for improved healing times and a better quality of life.

Currently available pharmacological treatments of osteoporosis are mostly anti-resorptive agents (e.g. bisphosphonates) which inhibit the action of osteoclasts and so preserve remaining bone mass (Poole & Compston 2006). Comparatively there has been little development of anabolic agents, which increase bone formation and aid bone mass recovery through osteoblast stimulation (Canalis et al. 2007). Currently, the only available anabolic agent is parathyroid hormone (PTH), which stimulates various osteoblast signalling pathways (Canalis et al. 2007). PTH is known to be effective in the treatment of severe osteoporosis,

though the cost of 1 months medication is roughly 10 times greater than alternatives (Lau et al. 2012) and is currently tightly regulated by the National Institute for health Care and Excellence (NICE). To address these limitations novel and recent research on anabolic agents has been conducted, such as the work of Negishi-Koga *et al.* (2011) on semaphorin 4D, a molecule expressed by osteoclasts. Semaphorin 4D was shown to bind to its osteoblast receptor, Plexin-B1, resulting in suppression of IGF-1 signalling and osteoblast motility. Negishi-Koga *et al.* showed that bone formation could be increased both through gene knockout and use of Semaphorin 4D specific antibodies, making this molecule a promising target for developing an anabolic osteoporosis treatment. Such research is promising and serves to highlight the need for more focused anabolic studies.

1.2.3 Osteopetrosis and Paget's disease of bone

Osteopetrosis, commonly called 'marble bone disease', is a rare and heritable disease characterised by increased bone density (Stark & Savarirayan 2009). It is caused by mutations in 10 genes (accounting for 70% of cases) which alter the differentiation and function of osteoclasts to reduce bone resorption (Stark & Savarirayan 2009). No effective remedy currently exists for this condition, though specialist fracture treatment (Farfán et al. 2015) and calcium/vitamin D supplementation are common. Paget's disease of bone (PDB) is also characterised by abnormal osteoclast activity (Kular et al. 2012); accompanied by hyperactive and irregular bone formation by osteoblasts, resulting in poor quality tissue. As with osteopetrosis the cause of PDB is thought to be genetic, though the exact source of the disease is unknown (White & Rushbrook 2013). However, unlike osteopetrosis the disease can be effectively controlled through the administration of bisphosphonates (Wat 2014).

1.3 Marine bioactives; their pathways, effects and potential

As mentioned, bioactive factors are the least developed member of the triad in terms of clinical application. This is no doubt influenced by the vast array of potential sources of these factors, e.g. from plant, animal or marine organism sources. Whilst there has been significant effort to identify potential bioactives, research is often constrained by the time and cost implications of exploratory work, not to mention conservational and yield issues which raise feasibility concerns (Proksch et al. 2003). For example, in a recent natural product review (Blunt et al. 2017) it was highlighted that a secondary metabolite isolated from the sponge *Spirastrella mollis* had an extremely low yield, limiting its identification and future use. The overall dearth of research does imply that novel work may identify potential new and possibly efficacious treatments for the clinical issues previously discussed. Marine organisms are a particularly promising area to search for such compounds, as they are exposed to an extreme range of environmental variables (i.e. nutrient, light and temperature levels) which act as a catalyst for specialization and adaptation (Jha & Zi-rong 2004; Smith et al. 2010). Furthermore, the difficulties and costs associated with exploiting this huge reserve of organisms means they have been even less well-studied than terrestrial taxa. Currently, marine bioactives can be easily sourced from commercial by-products, such as fish and shellfish remains (Harnedy & Fitzgerald 2013). In terms of our focus on invertebrates, by-product levels are much lower and whilst dedicated work on single species has been conducted, studies are still limited in number and understanding – see table 1.1 for overview of main species/extracts investigated to date.

Table 1.1: the genus, species, general description and extract type of key invertebrates that have been tested for their impact on bone cells or tissue. This is not an exhaustive list but demonstrates the range of taxa investigated within this field, many of which are referred to in the text. Numerous other marine species have had their molecular components determined, many of which show similarity to human skeletal proteins/growth factors (as discussed by Green *et al.* 2013). However, this list only incudes those extracts that have actually been tested in cell culture or relevant *in vivo* models.

Genus and species	**General description**	**Extract?**
Undaria pinnatifida	Brown algae	Fucoidan
Ascophyllum nodosum	Brown algae	Fucoidan (low molecular weight)
Sargassum horneri	Brown algae	Raw extract
Laminaria japonica	Brown algae	Fucoidan
Sargassum siliquastrum	Brown algae	Sargachromanol G
Sargassum thunbergii	Brown algae	Quinone derivatives
Hizikia fusiforme	Brown algae	Water by-product
Cladophora rupestris	Green algae	Crude extract
Codium fragile	Green algae	Crude extract
Laurencia undulata	Red algae	Floridoside
Lithothamnion corallioides	Calcareous red algae	Aquamin
Nannochloropsis oculata	Microalgae	Peptide
Alteromonas infernus	Prokaryote	Polysaccharide
Symploca sp.	Cyanobacterium	Largazole (depsipeptide)
Millepora dichotoma	Hydrocoral	Bioactive material
Porites lutea	Stony coral	Bioactive material
Porites lutea	Stony coral	Biomatrix
Synularia polydactyla	Alcyonarian coral	Proteins
Xenia elongate	Soft coral	Coral cells
Montipora digitata	Hard coral	Coral cells
Stichopus japonicus	Sea cucumber	Fucan sulfate
Haliotis discus hannai	Abalone	Digested intestines
Haliotis laevigata	Abalone	Perlucin protein
--	Mussels	Adhesive protein
Crassostrea gigas	Oyster	Protein Nacre (water soluble matrix)
Pinctada maxima	Pearl oyster	Individual proteins. Low molecular weight molecules.
Pteria martensii	Pearl oyster	Nacre (water soluble matrix)
Pinctada margaritifera	Oyster	Proteinase inhibitor. Proteins. Nacre (water soluble matrix).
Pinctada fucata	Akoya pearl oyster	Pinctada fucata mantle gene 3. Protein p10 and other novel proteins.

1.3.1 Current marine bioactive research

1.3.1.1 Algae

One group of marine invertebrates which show promise as a source of bioactives is algae, particularly macroalgaes. Within the algal group, fucoidan is one of the best studied extracts. Fucoidans are highly sulfated and fucose-rich polymers, found as a heavily branched and relatively high yield form in brown macroalgae and a more linear form in echinoderms (Fitton 2011). These marine polymers are multifunctional, with a range of therapeutic uses from anti-inflammatories and anti-virals (Fitton 2011).

In terms of its effects on bone tissue it appears that the extract has both an anti-resorptive and osteogenic potential. Fucoidan extracted from the sea cucumber *Stichopus japonicus* has been shown to inhibit osteoclastogenesis (Kariya et al. 2004), whilst work by Kim et al. (2014) demonstrated the same; though with respect to brown algae extracts added to bone marrow macrophage cultures. This reduction in osteoclast differentiation was attributed to inhibition of RANKL dependent MAPKs and down-regulation of c-Fos and NFATc1 transcription factors. However, the precise molecular mechanism through which this inhibition occurs is yet to be elucidated, which is a common limitation to studies on this polysaccharide. Despite this, it is speculated that fucoidan binds to either RANKL or RANK to inhibit intracellular signalling, an action which may be facilitated by its sulphated regions (Kim et al. 2014).

With respect to osteogenic potential, low molecular weight (LMW) fucoidan can promote osteoblast proliferation *in vitro*. This was measured as increases in the presence of osteoblast markers including ALP and type 1 collagen in 3D (S. Igondjo Tchen Changotade et al. 2008) and 2D culture (Y.-D. Cho et al. 2009; S Igondjo Tchen Changotade et al. 2008). The *in vitro* potential of fucoidan was also demonstrated in work by Pereira et al. (2014), whereby extract treatment increased osteogenic differentiation as measured by ALP, osteopontin, Runx2 and calcium deposit formation in adipose tissue-derived stem cells ADSCs. However, one limitation of current published studies is a lack of *in vivo* testing, which may simply be a function of the early developmental stage of much of this work. Pereira et al. (2014) did make efforts to address this, through use of coated biphasic calcium phosphate implants in mice, though no significant effect of fucoidan coating on bone formation was observed. This may indicate that fucoidan would be better applied to stimulate *ex vivo* osteogenic differentiation before *in vivo* re-implantation, though more testing is required to confirm this. Alternatively, it may be that the drug delivery method is important, and that direct injections or coupling with another form of scaffold, perhaps polyglycolic or lactic acid, would give better results.

To date the most comprehensive study completed on fucoidan is that of Kim *et al.* (2015), which aimed to elucidate the complex mechanism by which the extract stimulates MSC differentiation into osteoblasts. ALP, mRNA levels of multiple other markers and alizarin red-S (AR-S) staining was used to determine osteoblast differentiation and mineralisation, whilst western blotting was also conducted, including antibodies for various intracellular signalling pathway proteins. The results indicated that BMP2-Smad 1/5/8 signalling was responsible for stimulating osteoblast differentiation through activation of ERK and JNK pathways.

Whilst fucoidan is one of the better known bioactives there are other marine algae extracts which also have an effect on bone homeostasis. Extracts from the brown algae *Sargassum horneri* are known to stimulate osteoblastogenesis and inhibit osteoclastogenesis *in vitro* in preosteoblastic and monocyte cell lines (M Yamaguchi & Matsumoto 2012). Similar *in vitro* work was conducted using rat femoral tissues, which demonstrated the ability of *S. horneri* extracts to increase their bone calcium content (Uchiyama et al. 2004) and inhibit bone resorption (Uchiyama & Yamaguchi 2002). Such work complements *in vivo* tests, which indicate that *S. horneri* extracts have a preventative effect on bone loss in streptozotocin-diabetic rats (Uchiyama & Yamaguchi 2003). Furthermore, there has even been a basic human trial investigating the effect of oral intake of the algae on bone metabolic markers (Matsumoto et al. 2008). Despite being limited by very small sample sizes, this study reported an inhibitory effect of the extract on bone resorption, as determined by decreased levels of circulating resorption markers such as TRAP, and is one of the few human trial studies conducted for any marine based compound. As with fucoidan, the main limitation to the *S. horneri* body of work lies in identifying the active component of the extract, which appears to be different for bone stimulation and suppression of resorption (Uchiyama et al. 2004). Another brown algae extract which has been tested both *in vitro* and *in vivo* is *Hizikia fusiforme* (Jeong et al. 2016). Specifically, this study tested a hot water by-product of the algae which contained high polysaccharide levels and was shown to stimulate ALP activity and BMP-2 levels in mouse myoblast C2C12 cells. Furthermore, *in vivo* stimulations of skeletal activity were confirmed in zebrafish, ovariectomized mice and mouse calvaria bones (Jeong et al. 2016). A final brown algae based study investigated quinone derivatives from *Sargassum thunbergii*, finding that treatment with a crude extract containing these derivatives was able to enhance osteoblast differentiation (Kim et al. 2016).

In addition to Phaeophycae, green and red algae also show examples of extracts which are able to stimulate osteogenic activity. For example, extracts from two marine green macroalgae, *Cladophora rupestris* and *Codium fragile*, were shown to increase osteogenic

activity. These extracts were enriched in phenolic compounds and able to stimulate mineralogenic activity of a fish bone-derived cell line, as well as 1.5 fold increases in operculum area of juvenile zebrafish (Surget et al. 2017). Floridoside is the best example of a red algae derived osteogenic bioactive. This glycerol glycoside metabolite of *Laurencia undulata* (amongst other red algae) is known to promote differentiation of osteoblast D1 cells, as well as increasing ALP levels, mineralisation and expression of factors including type I collagen, Runx-2 and Osterix (Ryu et al. 2015).

The studies discussed appear to indicate that algae may be a promising reserve of bioactive compounds, whilst the ease of sourcing these inshore and shallow water eukaryotes makes them a likely commercial source. However, it is apparent that more *in vivo* studies are needed, as well as a focus on the mechanism of resorption inhibition and the active molecular component of algal extracts.

1.3.1.2 Aquamin

Aquamin is a food supplement derived from the red algae *Lithothamnion corallioides,* and contains calcium, magnesium and 72 other trace minerals (Gorman et al. 2011). Though an algae, *L. coralloides* has been included separately here due to its calcareous skeleton which distinguishes it from the previously discussed non-mineralised algal species. *L. corallioides* is normally found on muddy or sandy substrates at less than 20m depth, in aggregations of unattached algae known as maerl beds (Wilson et al. 2004). It is from these beds, with a wide European (including western Irish and British shores) and more northern distribution, that maerl is collected before it is ground into the commercial product known as aquamin. Currently, aquamin is solely licenced to and produced by Marigot Limited (Marigot, Cork, Ireland).

Mineralising organisms like *L. corallioides* are an obvious choice when searching for extracts to promote bone health due to their structural similarities, tendency to contain similar key molecules (David W Green et al. 2013) and high mineral content. The latter is the theoretical basis of aquamin, as there are well established links between BMD and the intake of minerals such as calcium, magnesium and zinc (Palacios 2006). From a cellular perspective O'Gorman *et al.* (2012) investigated the ability of aquamin to enhance the mineralisation of a pre-osteoblastic cell line. Mineralisation did increase, though only at the latest time point (day 28), whilst other markers of osteoblast activity, for instance proliferation measures, did not show significant differences between control and treatment. *In vitro* mineralisation is further

enhanced when vitamin D is included with aquamin treatment, as compared to levels in osteoblast cultures treated with only aquamin or control solutions (Widaa et al. 2014). These studies appear to indicate aquamin has potential to increase mineralisation, though further work is needed.

Aquamin was tested *in vivo* by inclusion in the food of mice given a western style diet, which is known to detrimentally impact bone strength and mineralization (Aslam et al. 2010). Aquamin was shown to negate diet related bone defects, further supporting its potential to enhance mineralisation. Alternatively, the supplement may be acting by increasing bone turnover rather than mineralisation, as supplementation studies on horses showed enhanced levels of osteocalcin and type I collagen relative to limestone controls (Nielsen et al. 2010). Interestingly, despite a lack of more basic studies, two randomised control trials have been conducted, aimed at assessing the efficacy of aquamin supplementation in treating knee osteoarthritis (Frestedt et al. 2009; Frestedt et al. 2008). These studies reported improvements in outcome measures of mean walking distance and range of motion for aquamin treatment compared to controls. However, these results are questionable due to the short study periods (12 weeks), lack of long term follow up and small sample sizes used. Overall, further *in vitro* and *in vivo* studies are required before the true potential of aquamin supplementation becomes apparent. However, irrespective of its treatment prospects *L. corallioides* is unlikely to be a long term resource, as it has a slow growth rate (1mm) and is highly susceptible to sedimentation through natural or anthropogenic means (Wilson et al. 2004).

1.3.1.3 Nacre

Another calcareous based extract, and one which is supported by a considerable body of research, is nacre. Nacre, also known as mother of pearl, is the lustrous aragonitic inner layer found on molluscan shells in taxa such as mussels and abalone. Like bone, nacre has both inorganic and organic components, with an organic shell matrix comprised of proteins, glycoproteins and polysaccharides which then serve as a template for calcium carbonate mineralisation (Marie et al. 2009).

Research on nacre has been conducted since the early 1990's, with initial *in vitro* work demonstrating its capacity to stimulate the mineralisation of human osteoblasts (Silve et al. 1992). The most interesting of these early studies investigated the ability of nacre to aid bone reconstruction in human maxillary defects (Atlan et al. 1997). Here, nacre was mixed with

the blood of patients before being injected into the defect site of eight middle-aged female patients. The results showed no evidence of toxic effect and also demonstrated enhanced mineralisation and good biodissolution of nacre within the area of injection (Westbroek & Marin 1998). Since this early work there has been a surge of research effort including *in vitro* and *in vivo* studies, as well as those specifically focusing on the proteins and mechanisms involved in enhancing cellular activity; making nacre an excellent case study of bioactive research.

A good example of the *in vitro* work conducted used water soluble matrix (WSM) extracted from the oyster *Pinctada fucata* (Chaturvedi et al. 2013). This study demonstrated both the ability of nacre to enhance osteoblast differentiation (increased Col-I, osteocalcin and ALP expression) and its ability to scavenge free radicals, suggesting an antioxidant potential that may also support bone regeneration. WSM has also been shown to increase BMD in an ovariectomized mouse model of osteoporosis (Kim et al. 2012), in part attributed to increased Runx2 and Fos-related antigen-1 expression as a result of JNK pathway stimulation in osteoblasts. Furthermore, the extract suppressed actin ring formation and RANKL- induced up-regulation of c-Fos and NFATc1 in osteoclasts. Other *In vivo* work showed nacre implants into rat femurs supported new bone formation, implant/bone fusion and increased expression of numerous markers indicative of increased BMU action (Liao et al. 2002).

As the osteogenic potential of nacre is well established numerous studies have worked towards identifying active components within the extract. At a basic level, nacre WSM is known to be made up of a variety of fractions, containing amino acids of varying size and composition (Almeida et al. 2000). Moreover, numerous proteins have been identified within the nacre of different species, many of which are thought to have roles in regulating bone tissue. For example, proteomic nacre analysis of the oyster *Crassostrea gigas* found four novel proteins thought to aid in shell mineralization; with structures homologous to endogenous human proteins also with roles in osteogenesis (Oliveira et al. 2012). Novel single proteins have also been identified, for example p10 (Zhang et al. 2006), P60 (Lao et al. 2007) and PFMG3 (Wang et al. 2011b), all sourced from the pearl oyster *Pinctada fucata*. All three of these studies demonstrated the proteins' ability to enhance the crystallisation of calcium carbonate *in vitro.* Each also established their ability to enhance the differentiation of osteoblast cell lines through various marker assays, though the work of Wang *et al.* (2011) was the most comprehensive. Additionally, nacre appears to contain proteinase inhibitors of varying molecular weights, which may help to conserve proteins important for processes such as mineralisation (Bédouet et al. 2007). The matrix also hosts low molecular weight

molecules, which were shown to increase osteoblast mineralisation, Col-1 expression and mRNA levels of Runx2 and osteopontin (Rousseau et al. 2008). Overall then, these studies indicate that the effect of nacre, regardless of species, is due to the interaction of a range of bioactive molecules present within the extract. However, the exact structure and action of the majority remain to be identified.

1.3.1.4 Assorted taxa

Corals, of the phylum Cnidaria and class Anthozoa, are another group which have received surprisingly little attention. This phylum includes mineralising species, which have a skeleton comprised of calcium carbonate either in the form of calcite, aragonite or a mixture of the two, as with the Scleractinian or stony corals (Rogers 1999). Like those taxa containing nacre, coral skeletons also include an organic phase, comprised of proteins, polysaccharides, lipids and glycosaminoglycans (David W Green et al. 2013). Marine invertebrates such as corals have a long evolutionary history of developing proteins and genes that govern biomineralisation, many of which are highly conserved and analogous to human variants (David W Green et al. 2013). For instance, in scleractinians both soluble and insoluble components of the organic matrix are known to influence calcium carbonate precipitation (Goffredo et al. 2011). More specifically, multiple species express BMP2/4 orthologs, which show specificity to murine BMP receptors despite the taxonomical distance between the two groups (Zoccola et al. 2009). This indicates incredible conservation of the protein sequence over time and suggests other proteins of skeletal importance may also be present within coral tissue. The presence of these bioactives is also supported by the regeneative ability of corals, many of which show relatively rapid skeletal repair, particularly those species living in shallow water, high-energy environments (Kramarsky-Winter & Loya 2000). Despite their significance as a potential source of bioactive molecules, research using coral extracts is limited. Instead, the majority of research has focused on their potential as a scaffold material for graft procedures, due to their structural and compositional similarities to human bone (Clarke et al. 2011).

There are numerous examples of taxa and extracts that have only undergone initial screening, many of which show osteogenic potential. For example, the *in vitro* potential of abalone gastro-intestinal digests from *Haliotis discus hannai* were investigated using an osteoblast-like cell line (MG-63), and subsequently showed increased ALP and mineralization levels (Nguyen et al. 2014). RT-PCR and western blot analysis found increased BMP-2

expression, thought to be a result of MAPK pathway activation. Similar results are presented for a range of extracts, even for microalgae byproducts (Nguyen et al. 2013) and a depsipeptide (largazole) sourced from cyanobacteria (Lee et al. 2011). Another interesting prokaryote is *Alteromonas infernus*, which is a deep sea species known to produce a high molecular weight polysaccharide. This oversulfated polysaccharide was shown to increase chondrogenesis *in vitro*, via the MAPK pathway, and may consequently be a potential therapy for cartilage repair (Merceron et al. 2012). Mussel adhesive proteins are also of interest as they are known to increase cell proliferation and osteogenic differentiation, and can be used to easily coat graft materials (Hong et al. 2012). This, along with their biocompatibility and biodegradability, makes them promising adjuncts to synthetic grafts. These preliminary studies, looking at various markers of cell-stimulation potential, highlight the diversity of marine bioactives and indicate that it is primarily research effort which determines success within this field.

1.3.2 Challenges

Conservation concerns are an important consideration when looking for potential bioactive sources. Taking corals as an example, it is well known that the world's populations are a serious conservation issue (Hughes et al. 2010), with both cold and warm water species showing declining abundance and many listed in the convention on international trade in endangered species (CITES) list (Harriott 2003). Reasons for the decline are varied, including increasing water temperature and ocean acidification associated with climate change, as well as damage from trawling, dynamite fishing, aquarium stocking and various forms of anthropogenic pollution (Hughes et al. 2010). This, coupled with slow growth rates, long time to maturity and relatively limited ecological niches, means further declines in coral numbers are likely. The sustainability of these invertebrates as a source of bioactives is therefore questionable, although some species like *Millepora dichotoma* are more abundant and show promise for aquaculture production (Abramovitch-Gottlib et al. 2006). Though corals are perhaps an extreme example in terms of sustainability, many marine invertebrates suffer from low abundance or long development cycles. In these cases bacterial cultures (modified to express the bioactive) or synthetic production are potential solutions, though complex molecule structures often make this challenging (Proksch et al. 2003).

Aquaculture may become a necessity if commercial bioactives are to be sourced from marine organisms, as yields of target compounds are often very low, needing a large quantity of raw

material for significant extraction (Proksch et al. 2003). In particular, secondary metabolite yields tend to be very low, though structural and non-metabolic proteins (like many of those with skeletal importance) tend to have higher yields (David W Green et al. 2013). It is not just a question of tissue yield but also the costs and difficulties associated with collecting enough quality specimens in the first place, an issue which is further complicated when sourcing deep sea species. Advances in technology, like the development of sophisticated remote operated vehicles (ROVs), is beginning to make collection of these specimens more feasible.

From a more general perspective there are also a number of limitations to more widely available bioactives, growth factors and other therapies. For example, as mentioned previously, BMP 2/9 are currently available for clinical use in the UK and US. However, trials involving these bioactives have been limited to date and as a result their efficacy is still debated (Garrison et al. 2007), despite usage becoming more common. Alternatively, hormones such as teriparatide can be used to stimulate anabolic action of the BMU. Whilst effective, treatment courses are currently long, expensive and require daily injections, with any positive effects quickly lost after ceasing treatment (Black & Rosen 2016; Canalis et al. 2007). One interesting and developing treatment option is gene therapy, which involves the transfer of target genes which code for proteins which stimulate bone formation/regeneration. This therapy has already been shown to aid bone healing, though there are several issues with its implementation (Balmayor & van Griensven 2015). For example, therapy may also stimulate the immune system which can interfere with treatment, whilst in rare cases tumours can form because of poor DNA integration. Furthermore, if viruses are used for gene transfer there are also toxicity and disease concerns that need to be addressed.

1.3.3 Summary

Throughout this narrative the complexity of bone, its remodelling and the myriad of effects bioactives can have on cells of the BMU have been introduced. Remodelling encompasses a host of different cellular activities in a complex series of events, which can be influenced in multiple ways by localised factors. For example, a bioactive may serve to stimulate the recruitment and commitment of MSCs to the osteoblast lineage, as with nacre powder (Flausse et al. 2013). Similar anabolic bioactives may increase the proliferation of progenitor cells leading to greater numbers of osteoblasts carrying out bone formation. Alternatively, an extract could impact more mature cells, leading to increased cell differentiation or

mineralisation, as shown during aquamin treatment (Widaa et al. 2014). However, in addition to promoting osteoblast activity, a promising extract might also limit osteoclastogenesis and the activity of these cells. The complexity of this situation increases when using a raw, whole or relatively unrefined extract, as the material may contain any number of bioactives having different or competing effects. When working with extracts to identify novel compounds it is therefore important to determine their primary effects, using both *in vitro* and *in vivo* techniques, and if promising also clinical trials. Two of the most challenging aspects of this work are isolating the active compound(s) and determining the mechanism of effect, such as the intracellular signalling pathway being stimulated or repressed. In addition to all of these considerations the source taxa also needs to be sustainable, have a high abundance, produce large extract yields or be suitable for aquaculture.

In this body of work a large number of extracts will be screened for their osteogeneic activity. This will be primarily focused on measures of human foetal osteoblast (hFOB) activity (cell line), with the basic screen measuring cell proliferation and ALP activity (differentiation measure), whilst also ensuring no cytotoxicity of potential treatments. After determining promising extracts a more detailed screen of osteoblast activity will be conducted, using human bone marrow derived stromal cells (hBMSCs). This is the best *in vitro* method of assessing osteogenic potential (David W Green et al. 2013), as cell lines are not fully representative of a human tissue response. Subsequent to this body of work the *in vivo* action of extracts will be determined, using zebrafish as a model for skeletal growth through a partnership at the University of Algarve. This diverse range of experiments will allow varying forms of osteogenic activity to be identified at different developmental stages, and is thus the most appropriate approach when searching for novel bioactives.

Chapter 2

Method development and optimisation

2.1 Introduction

Before starting any experiments, it is important to choose appropriate methods - which have the best chance of providing useful and reliable results. It is also crucial to properly analyse and optimize these methods, to ensure they are accurate, precise and therefore suitable choices. This chapter contains basic protocols for many *in vitro* techniques used throughout this thesis, on both a human foetal osteoblast (hFOB) cell line and human bone derived marrow stromal cells (hBMSCs). It also contains highlights from a significant body of optimisation studies for both cell types.

Work presented here (summarised in figure 2.1) was mainly completed during year one of the PhD, as issues with different methods became apparent. However, some optimisation spanned the entire three years, whilst other assays – particularly those focusing on cell differentiation – were not used until later in the project. Any protocols listed in section 2.2 are final, fully optimised versions, whilst remaining chapter sections explain how and why these were developed, as well as detailing other important cell culture decisions.

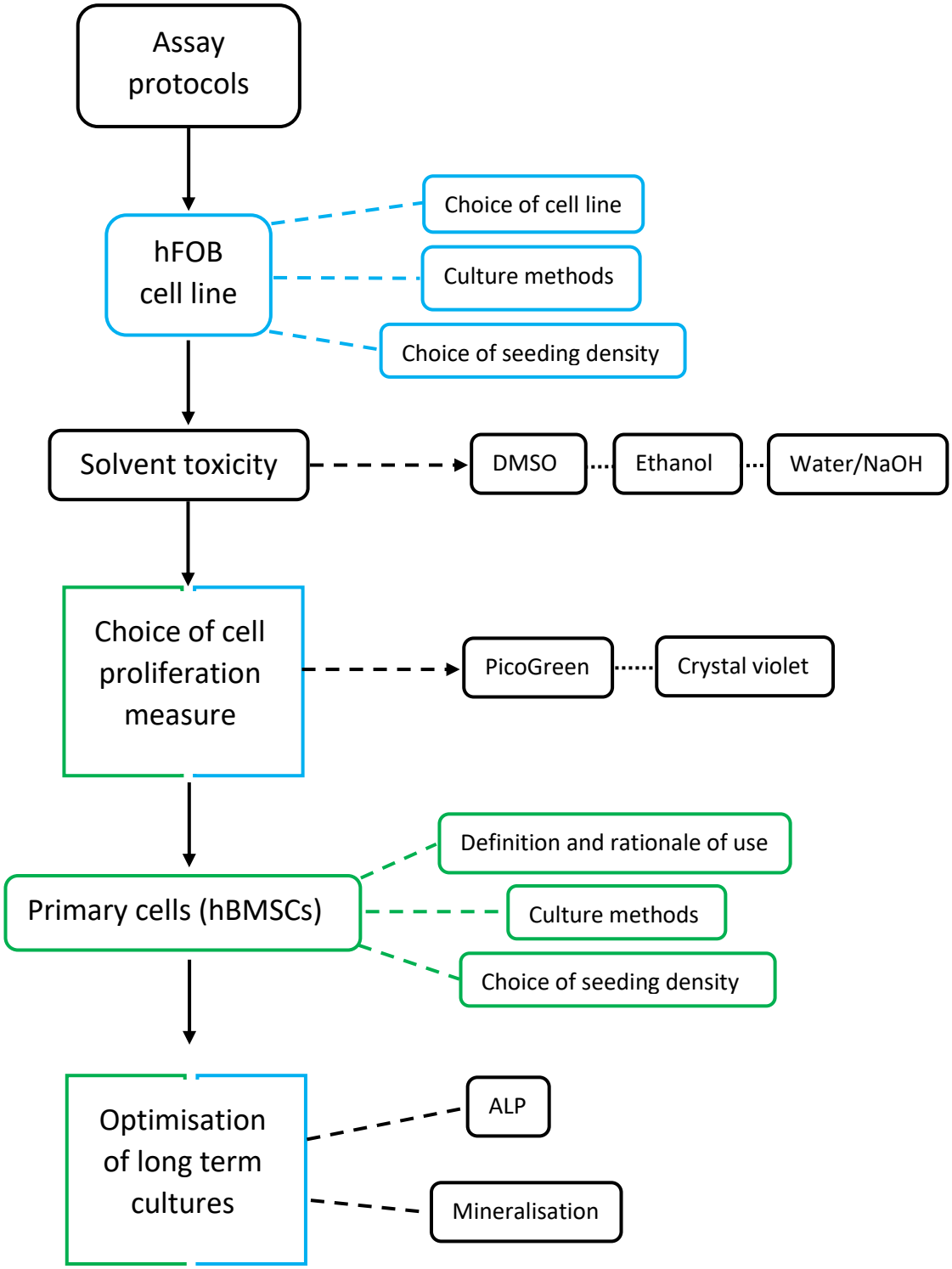


Figure 2.1: flow diagram detailing the sections/methods discussed in this chapter. Blue colouration indicates hFOB focused work and green hBMSCs.

2.2 General *in vitro* methods

2.2.1 XTT – cell viability

To quantify the number of viable cells the *In Vitro* Toxicology Assay Kit XTT based, or 'TOX2' kit, was used (Sigma Aldrich, UK). The method is simple to perform and gives an indication of the cytotoxicity associated with a treatment, as well as the degree of metabolic activity.

XTT (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) is yellowish in colour when first added to cell solutions and through normal cell metabolism, mitochondrial dehydrogenases reduce the tetrazolium ring to produce a red and soluble formazan product (Riss et al. 2004). After incubation, absorbance in each active well can be measured to determine formazan levels and therefore give an indication of metabolic activity levels. The assay was performed according to the manufacturer's instructions. Briefly, the assay substrate was reconstituted in culture medium and 40 µl, representing 20% of the total volume, was added to each active well on the culture plates. After incubating for 4 hours, the wells were thoroughly mixed and a 100 µl aliquot was transferred to a clean assay plate. Absorbance was read at 450 nm on a Tecan microplate reader with Magellan software (GENios, USA).

2.2.2 Lactate dehydrogenase – cytotoxicity

The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega) was used to quantify the amount of lactate dehydrogenase present within a sample. This enzyme is constrained to the cytoplasm of healthy cells with preserved membranes, but is released into the culture medium from dead or dying cells (G. Wang et al. 2012). As such, it is a good measure of the cytotoxicity of a substance/treatment. This assay benefits from being able to detect low level cytotoxicity and is therefore suitable for use after short culture periods, such as the day 1 time points used in this thesis.

The assay works through a coupled enzymatic reaction which takes place over a 30-minute interval. Simply, those cells that are damaged release LDH, which then catalyses the conversion of lactate to pyruvate, via the reduction of NAD+ to NADH. Concurrently, the enzyme diaphorase utilises NADH and tetrazolium salt (INT) to produce NAD+ and formazan, a dye product which is red in colour. The amount of formazan and therefore the degree of colouration is directly related to the LDH level, giving an indication of cell death.

In general, cells were given a 24-hour attachment period and 24 hours (or sometimes 4 days) of exposure to treatment, before conditioned medium was removed and LDH level quantified according to the manufacturer's instructions. Firstly, 50 µl of conditioned medium was added, in duplicate, to test plates. Negative controls were medium and medium+vehicle. Positive controls were a kit control and lysate from an aliquot of a known amount of cells, which had been pre-treated with 10 µl lysis solution (9% v/v Triton X-100) per 100 µl cell media. The assay substrate was reconstituted in buffer solution and 50µl was also added to each well. Each test plate was then covered in foil and incubated for 30min at room temperature, allowing the coupled enzymatic reaction to proceed. The reaction was stopped by the addition of 50 µl of stop solution (1 M acetic acid) to each well, before absorbance was read at 492 nm.

2.2.3 PicoGreen – cell proliferation

The Quant-iT™ PicoGreen® dsDNA Assay Kit (Molecular Probes 2008) is used to quantify the amount of double stranded DNA (dsDNA) present within a sample. This is accomplished through use of a PicoGreen reagent, which stains for nucleic acids.

Following 1, 4 and 7 days' exposure to extracts, medium was removed and the cells were washed x 2 with PBS or alkaline buffer solution (5 M NaCl, 1 M Tris-Cl pH 9.5, 1 M $MgCl_2$). 250 µl of lysis buffer (0.2 % Triton X-100 in PBS or alkaline buffer) was added to each well which was then subjected to at least 1 cycle of freeze (-80°C) and thaw (+37°C). This ensured that cell membranes were completely lysed through ice crystal formation, therefore releasing all dsDNA into solution. Sample wells were then mixed and 50 µl of each lysate was added to black assay plates in duplicate. A standard curve of 1 mg DNA/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml and 0 ng/ml was added in duplicate to each assay plate. Finally, 50 µl of PicoGreen reagent was added to all wells and fluorescence levels were read at excitation 480 nm, emission 520 nm.

2.2.4 Crystal violet – cell proliferation

Crystal violet (CV) stains for adhered cells by binding to DNA and proteins, and was used in this thesis to measure cell proliferation (Feoktistova et al. 2016). Cells were cultured in test plates for either 1, 4 or 7 days before the assay was completed. Culture medium was first removed by aspiration. Subsequently, cells were fixed through a 30-minute incubation at room temperature after addition of 100 µl of 2% paraformaldehyde (PFA) solution (pH 6.8 in

PBS). This fixative was then removed, test wells were washed 2x with dH_2O and left to air dry. Cell monolayers were stained for 30 minutes at room temperature using 100 µl of CV solution per well (at 0.1% concentration, in dH_2O and filtered before use). After staining, the CV was removed, the monolayer was washed 2x with dH_2O and air dried. Finally, this dye was extracted from monolayers by the addition of 100 µl of 1 M acidified methanol (1 ml of 1 M HCl per 100 ml methanol). Plates were read at 585 nm and blanked on acidified methanol.

2.2.5 Alkaline phosphatase – cell differentiation

Alkaline phosphatase (ALP) is an enzyme produced by osteoblasts, which catalyses the conversion of p-nitrophenyl phosphate to p-nitrophenyl, which is yellow in colour. This colour change can then be quantified giving an indication of alkaline phosphatase activity and therefore cell differentiation. Briefly, cells were grown for 7, 14 and 21 days, before being washed with an alkaline buffer solution (5 M NaCl, 1 M Tris-Cl pH 9.5, 1 M $MgCl_2$). Cells were then lysed by addition of 250 µl of buffer - containing 0.2% Triton X-100 - and left to gently mix for 20 minutes on ice, before being stored at -80°C. Upon defrosting, 50 µl from each well was added to a test plate in duplicate. 200 µl of conditioned medium, consisting of alkaline buffer solution (Sigma-Aldrich, UK) and p-nitrophenyl phosphate substrate (1 mg/ml; Sigma-Aldrich, UK), was added to each well. Each test plate was then covered in foil and incubated for 30 minutes at 37°C, allowing the coupled enzymatic reaction to proceed. The reaction was stopped by the addition of 50 µl of stop solution (3 M NaOH) to each well before absorbance was read at 450 nm. Finally, ALP readings were normalised to DNA concentration in the same lysate, determined via PicoGreen assay, to account for variances in cell number.

2.2.6 Mineralisation – cell differentiation

Mineralisation is a feature of mature osteoblasts, as the cells produce factors which control mineral production (Matsuo & Irie 2008). To detect this, cells were cultured in osteogenic supplemented media for either 7, 14, 21 and 28 days (in 24 well plates, first coated with excess 0.1mg/ml Poly-L-lysine [70,000-150,000 molecular weight] solution). Subsequently, they were washed 3x with PBS (1 ml) and fixed with 4% PFA for 1 hour at room temperature. Fixative was then removed and wells washed 3x with MiliQ water (1 ml). 40 mM alizarin red S, adjusted to pH 4.2 using ammonia hydroxide, was then added to each well (0.5 ml). Plates were left to stain at room temperature for 15 minutes, with gentle agitation on an orbital shaker. After staining the solution was discarded and each well was washed again, 4x with

MiliQ water, before being left to air dry. Finally, wells were de-stained using a solution of 10% cetylpyridinium chloride in sodium phosphate (pH 7). A 100 µl aliquot of each treatment was collected, transferred to a 96 well plate and then used for measuring absorbance at 550 nm.

2.3 Human foetal osteoblast cell line

2.3.1 Choice of cell line

Many experiments within this thesis, particularly preliminary ones, were conducted on the hFOB 1.19 (ATCC® CRL-11372™, Virginia, US) cell line. This cell line was originally established from biopsies of a spontaneous miscarriage, by transfection of primary cultures with a gene coding for a temperature sensitive mutant (tsA58) of the SV40 large T antigen (Harris et al. 1995). These cells were also transfected with a gene coding for neomycin (G418) resistance, resulting in a cell line that could be positively selected for by culturing with antibiotics; in this case geneticin, which would otherwise disrupt ribosome action and protein synthesis. hFOBs were shown to proliferate rapidly at the permissive temperature of 33.5°C, whereas at 39°C, division was greatly reduced and cell differentiation increased. Osteoblast phenotype was also confirmed through expression of osteonectin, osteopontin, bone sialoprotein, type I collagen, alkaline phosphatase and the osteoblast specific transcript osteocalcin (Harris et al. 1995). Further characterization showed that hFOBs had anomalies in only 1-2 chromosomes, were able to form extracellular matrix *in vitro* and were capable of *in vivo* bone growth in a mouse model (Subramaniam et al. 2002).

Since their characterization, hFOBs have been used for a variety of different applications, such as evaluating the mechanism of established bone regulators, like BMP-2 and TGF-β (Eichner et al. 2002), as well as anti-resorptive bisphosphonates (Reinholz et al. 2000). Furthermore, hFOBs have been used to assess more novel treatments, like the osteogenic potential of plant derived Osthole (Kuo et al. 2005) and Myricetin (Hsu et al. 2007), as well as marine organism inspired scaffolds for tissue growth (Clarke et al. 2016). Myricetin (a flavonoid compound) is a particularly interesting example, as it was tested on both MG-63 and hFOB cells to compare their responses (Hsu et al. 2007). Both cell types were almost identical in their response to myricetin, with broad trends always maintained and only slight differences in values between the two.

As discussed, hFOBs benefit from expressing key osteoblast markers, *in vivo* bone growth capability and limited chromosomal damage even after multiple passages. However, it should be noted that other osteoblast-like cell lines such as MG-63 cells, derived from an

osteosarcoma, could have also been chosen (Billiau et al. 1977). MG-63 cells show a sequential profile of osteoblast marker expression (Quarles et al. 2009), though proliferation and alkaline phosphatase activity is not very representative of bone cell cultures (Clover & Gowen 1994). Furthermore, MG-63 cells have a greater number of chromosome abnormalities than hFOBs (Subramaniam et al. 2002). Another option as cell choice was MC3T3-E1 (Sudo et al. 1983), a clonal osteogenic cell line derived from mouse calvaria, which shows mineralization potential and is frequently used in studies within this field. A review of *in vitro* osteoblast cell lines, not including hFOBs, highlights that each line has its own distinct advantages and disadvantages (Czekanska 2012). For example, MG-63 cells show no interspecies differences compared to MC3T3-E1, though have inconsistent levels of cell mineralisation. Furthermore, the MC3T3-E1 cell line is known to contain different subclones, which vary greatly in their mineralisation potential (Wang et al. 1999). As such, compared to the other cell lines available hFOBs were a good choice for *in vitro* screening, though no cell line can ever fully replace the use of primary cells (see section 2.6 b for further discussion). However, there are disadvantages to using primary cells in that they are more difficult to source and can only be used for a limited number of passages; therefore, hBMSCs were saved for testing those extracts with definite osteogenic potential.

2.3.2 Culturing hFOBs

2.3.2.1 Flask culturing

For normal cell growth, hFOBs were cultured in DMEM/HAM F12 (Sigma-Aldrich, 11039-021) medium containing 10% foetal bovine serum (FBS, PAA and Sigma-Aldrich after October 2016 – F7524, Lot 094M3341, UK), 2 mM L-glutamine (M11-004, PAA, UK) and 0.3 mg/ml of the selective antibody Geneticin (10131027, Gibco, UK). To encourage cell differentiation this media was supplemented with 50 µM ascorbate-2-phosphate and 10 µM β-glycerophosphate. Cells were cultured in standard T75 flasks, kept in a humidified incubator which was set at 33°C with a 5% CO_2 concentration. During culturing medium was replaced twice weekly. When cells reached between 75-80% confluency they were split in a ratio not exceeding 1:4, using 0.25% trypsin/EDTA (Gibco, UK) and a 2-minute incubation period to detach cells. When the desired quantity of cells were ready, they were trypsinised for use in experiments.

2.3.2.2 Plate culturing

Cells were used for experiments when approximately 80% confluency was reached in flasks. Cells were detached [as in 2.3.2.1], homogenised, an aliquot taken for counting and the remainder spun in an ALC 4218 centrifuge (Thermo Scientific, UK) for 10 minutes at 439 g. To count, cells were stained with trypan blue solution (Sigma-Aldrich, T8154) in a 1:2 ratio and counted using a Neubauer haemocytometer. Finally, cells were resuspended at the correct density and 200 µl of this cell solution was transferred to each well of a 96 well plate.

After plating out cells were always given a 24-hour attachment period, before media was changed for treatment media. Subsequently, cells were fed twice weekly until experiments ended.

2.3.2.3 Cryopreservation

Cryopreservation was also performed at 80% confluency. Cells were detached, counted with a neubauer haemocytometer and then added to the correct volume of cryopreservation solution (8-parts FBS, 1-part DMSO, 1-part complete hFOB media), to give a solution of approximately 1 million cells/ml. 1 ml of this solution was then added to each cryopreservation tube (Sial 0659, Sigma-Aldrich, UK), and cooled at a rate of 1 degree per minute until -80°C was reached. Cryopreservation tubes were then transferred to liquid nitrogen storage.

2.3.3 Optimisation of seeding density

Figure 2.2 shows some recurrent trends between treatments at different cell densities and time points. In general, addition of 0.1% DMSO to culture medium appears to cause a small increase in proliferation, except at the $0.5x10^4$ cells/cm^2 density at day 7, and $1x10^4$ cells/cm^2 at day 1. Treatments are more variable in their trends, though MR-05 frequently promoted increased dsDNA concentration (see chapter 3 for more detail). In terms of cell density, $1x10^4$ cells/cm^2 had decreased dsDNA concentration compared to $5x10^4$ cells/cm^2, as expected, though the two lower densities were more comparable - particularly at days 4 and 7. Most importantly, by day 7 values from the 50,000 cells/cm^2 density were above the maximum value of the standard curve, and have therefore been excluded from figure 2.2 C). Based on these results, a 10,000 cells/cm^2 seeding density was chosen for future hFOB experiments.

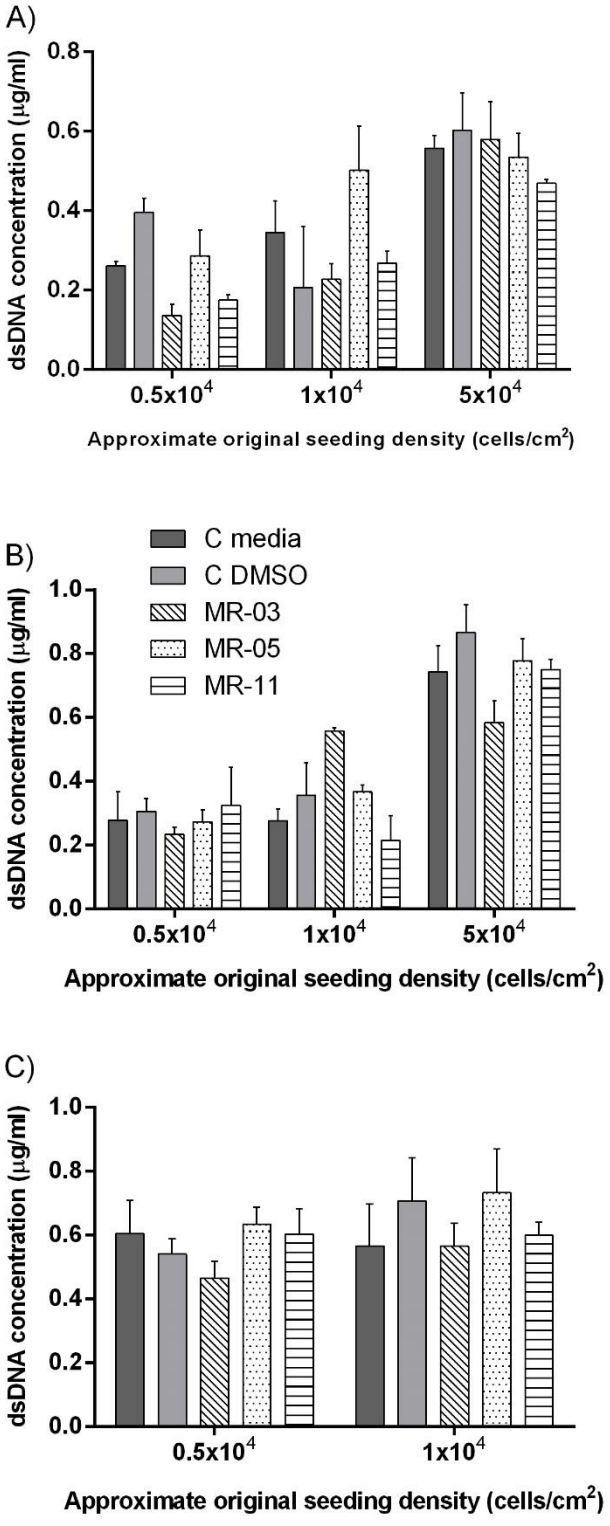


Figure 2.2: results from a PicoGreen assay conducted on hFOBs at day 1 [A)], day 4 [B)] and day 7 [C)] (after an initial 24-hour attachment period). Cells were cultured at three different densities: $0.5x10^4$, $1x10^4$ and $5x10^4$ cells/cm^2. Controls for both plain media and DMSO are included as well as three test treatments (MR-03, 05 and 11). Results are presented as the mean +/- standard deviation (n=4).

2.4 Solvent toxicity

2.4.1 DMSO and ethanol

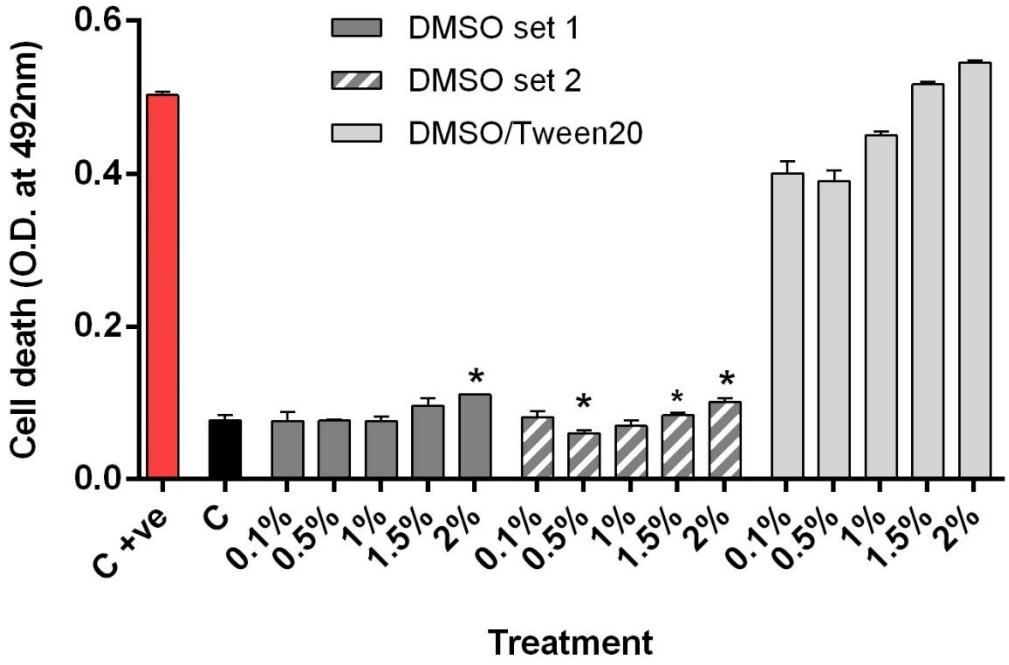


Figure 2.3: Results from an LDH assay conducted on hFOBs, where two sets of cells were treated with DMSO (denoted DMSO set 1 and 2) and one set with a 50:50 DMSO and Tween-20 mix (denoted DMSO/Tween20). Concentrations included: 0.1, 0.5, 1, 1.5 and 2% diluted in complete media. Cells were plated at a $1x10^4$ cells/cm^2 density, given 24 hours to attach, 24 hours with treatment and then supernatant was collected for testing. Results are presented as the mean +/- standard deviation (n=4). C+ve shows the absorbance values for 100% cell death. All values are corrected for background absorbance by subtracting appropriate negative control values. Kit positive control confirmed the assay worked for each test. *$p<0.05$ for treatment compared with medium only control.

Extracts supplied for this project were originally produced by a DCM/MeOH extraction of sample material by the MI (see chapter 3 for full description) and were supplied as a concentrated oily residue. DMSO was chosen to reconstitute these extracts during initial experiments because of its universal use and ability to dissolve a wide range of compounds (Kolář et al. 2002). DMSO is an organic, polar and aprotic solvent, with a large dielectric constant and dipole moments – traits which make it a highly efficient solvent. During early work DMSO concentration was limited to 0.1% dissolved in culture medium, as at higher concentrations it can induce changes in cell morphology and membrane properties (Du et al. 2006). However, to justify this choice and potentially raise extract concentration (and therefore likelihood of an effect), the impact of increased DMSO concentration was also investigated. Figure 2.3 indicates that 2% DMSO caused a significant increase in cell death compared to a complete medium control at day 1, whilst 1.5% is also slightly increased.

However, lower concentrations of 1, 0.5 and 0.1% show no apparent toxicity at this time point. Also, included in this figure was a 50:50 mixture of DMSO and Tween-20, to establish if this was a possible alternative solvent. Unfortunately, addition of Tween-20 caused substantial cell death, similar to or exceeding that of the positive control, even at 0.1%.

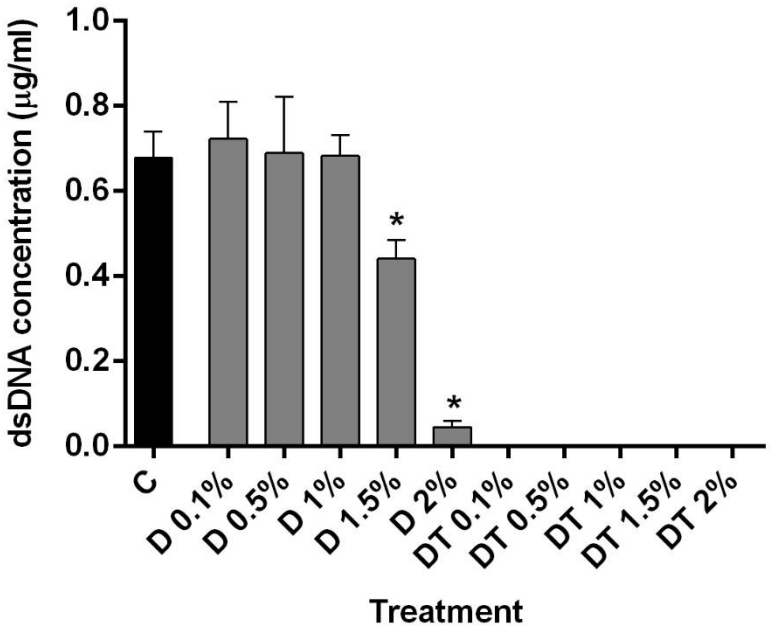


Figure 2.4: Results from a day 7 PicoGreen assay conducted on hFOBs, where cells were treated with either DMSO (denoted D) or a 50:50 DMSO and Tween-20 mix (denoted DT). Concentrations included: 0.1, 0.5, 1, 1.5 and 2% diluted in normal hFOB culture medium. Cells were plated at a $1x10^4$ cells/cm^2 density, given 24 hours to attach and 7 days with treatment. Results are presented as the mean +/- standard deviation (n=6 for control, n=4 for treatment), *p<0.05 for treatment compared with medium only control.

In addition to LDH testing, a day 7 PicoGreen assay was also carried out. Figure 2.4 again clearly shows that Tween-20 is toxic, with no discernible dsDNA present for any Tween-20 treatment; indicating complete cell death. On the other hand, DMSO has dsDNA present at all concentrations, though it is lowest at 2% and also significantly reduced at 1.5%. This complements cell death trends seen in figure 2.3, and suggests that high DMSO concentration causes a sustained increase in cell death over time. Based on these results it was concluded that 1% DMSO was suitable to include with cells over a 1-week experiment. Subsequently, a 7-day screen of 26 extracts was conducted, with each being included at a 1% concentration. However, (CV) had replaced PicoGreen as an assay at this point, and its results clearly showed a decrease in cell number with 1% DMSO, compared to plain medium controls. To investigate this further a smaller experiment was conducted, including controls and extracts at 1 and 0.5% concentrations. Results from this, presented in table 2.1, show that DMSO had a negative impact on cell proliferation at both 0.5 and 1% concentration, an

issue which worsened with time. It is for this reason results from these two extract screens have not been included in this thesis. Furthermore, these results served to highlight a marked difference between PicoGreen and CV proliferation values, creating concerns with PicoGreen as a method (see section 2.5 for discussion).

Table 2.1: the percentage decrease in CV proliferation value of DMSO treated cells compared to plain media controls, over days 1, 4 and 7. Included are values for cells at 0.5 and 1% DMSO concentrations

	% decrease in CV proliferation value - solvent compared to plain media control		
DMSO concentration (%)	**Day 1**	**Day 4**	**Day 7**
0.5	0	13	25
1	10	34	44

To better establish toxicity trends a second control experiment was performed, this time including LDH and CV assays. LDH (figure 2.5) results were like those of figure 2.3, showing low toxicity values for DMSO treatments that start to increase slightly at 1.5 and 2% concentrations; however, this time they were not significantly increased compared to the control. Ethanol, a polar protic solvent also with high dielectric constants and dipole moments, was also included in this study, as a potential replacement for DMSO if it proved too toxic for long term use at higher concentrations. As with DMSO, ethanol also appeared to show a very slight dose dependent response (figure 2.5), though all treatments had significantly lower cell death values than the control.

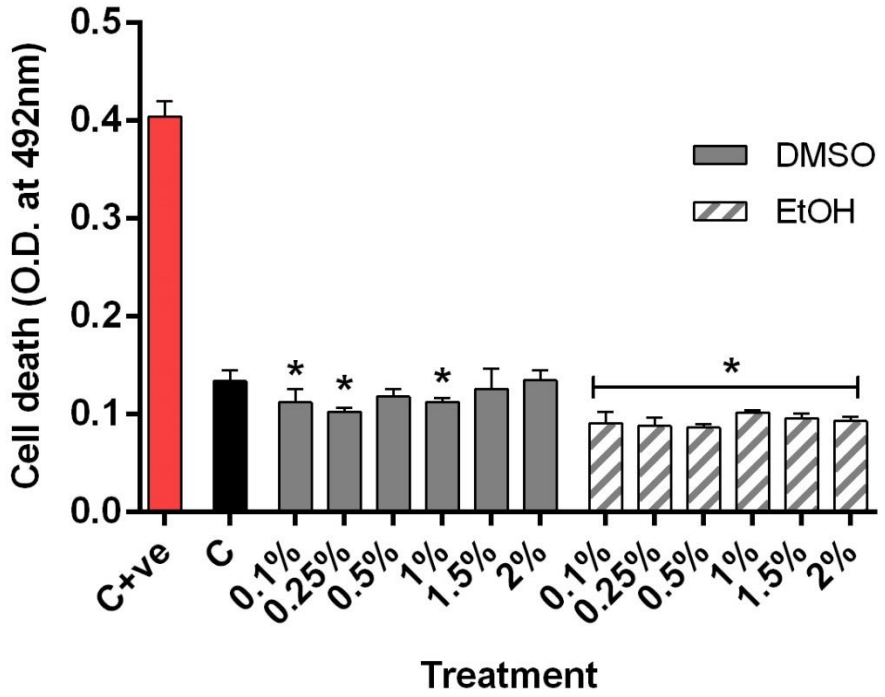


Figure 2.5: Results from an LDH assay conducted on hFOBs, treated with either DMSO or EtOH. Concentrations included: 0.1, 0.5, 1, 1.5 and 2% diluted in normal hFOB culture medium. Cells were plated at a $1x10^4$ cells/cm^2 density, given 24 hours to attach and 24 hours with treatment. Results are presented as the mean +/- standard deviation (n=2, duplicates). C+ve shows the absorbance values for 100% cell death. All values are corrected for background absorbance by subtracting appropriate negative control values. Kit positive control confirmed the assay worked for each test. *$p<0.05$ for treatment compared with medium only control.

For CV (figure 2.6), day 1 proliferation values are all quite similar, though 1.5 and 2% DMSO did cause a small yet significant decrease compared to the control. By day 4 these decreases are more pronounced and a reduction in cell number was also seen with 1% concentration. Ethanol showed no apparent decreases in proliferation at day 4, and instead a small significant increase in cell proliferation at 0.1 and 0.25%. By day 7, the increase at 0.25% EtOH is lost, though 0.1% is still significantly increased. Furthermore, cell number in 1.5 and 2% EtOH are now significantly reduced compared to the control, as are DMSO concentrations of 0.5% and above. These results are different to those of seen at day 7 with PicoGreen (figure 2.4), as the latter only indicated that 1.5 and 2% DMSO concentrations have a negative impact on proliferation. Alternatively, EtOH appears to have lower cytotoxicity than DMSO and should therefore be suitable for inclusion with cells up to a 1% level. Overall, discrepancies between PicoGreen and CV proliferation values are particularly interesting, as they indicate CV is the more sensitive technique. Their exact cause is unclear, but may be caused by differences in methodology and repeatability between the tests, or a whole range of PicoGreen issues (see section 2.5 for discussion). However, they do clearly highlight the

importance of establishing basic experimental design (such as solvent toxicity), not just between cell types or time points, but also different assays.

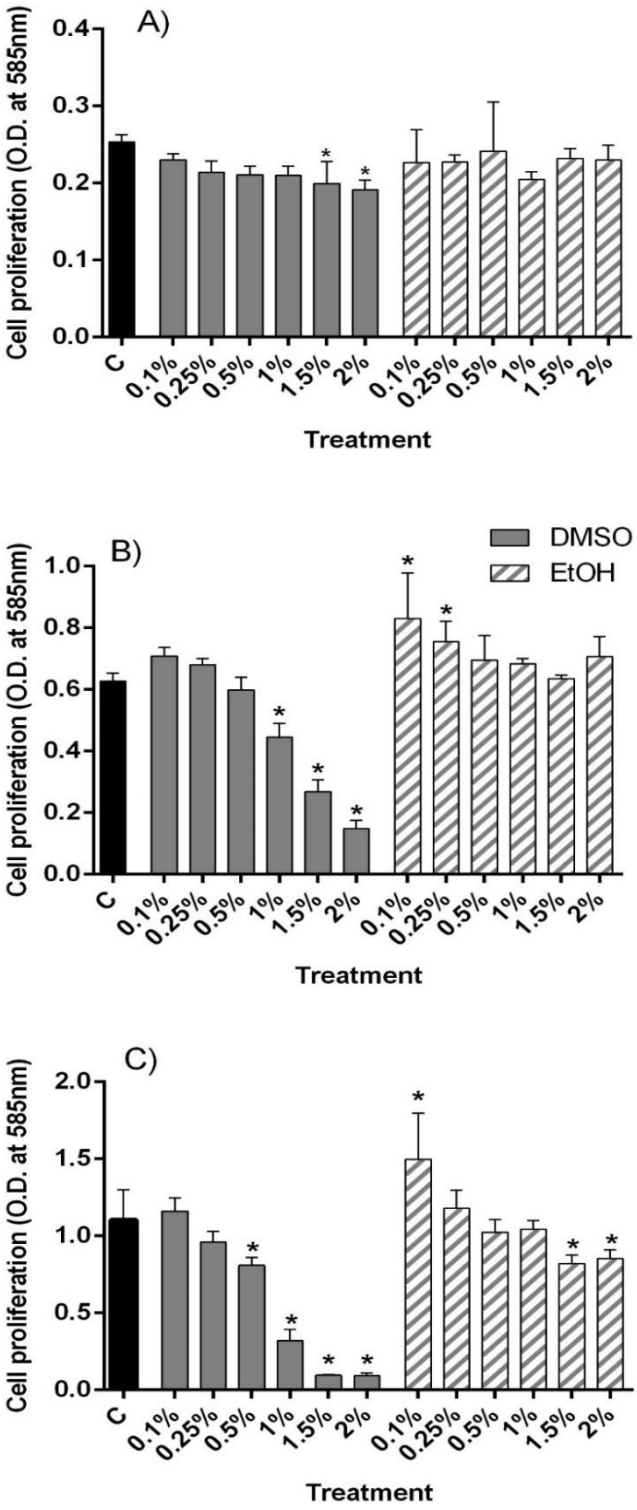


Figure 2.6: results from CV assays performed on hFOBs. Cells were plated at a $1x10^4$ cells/cm^2 density, given 24 hours to attach and then cultured for either 1 [A)], 4 [B)] or 7 [C)] days before being stained. Cells were treated with either a complete media control (C) or DMSO/EtOH, at concentrations ranging between 0.1 and 2% dissolved in culture media. Results are presented as the mean +/- standard deviation (n=4), *p<0.05 for treatment compared with medium only control.

2.4.2 Alkaline and aqueous extractions for powder extracts

After testing for osteogenicity in extracts reconstituted in DMSO and ethanol, powder extracts were also screened for activity. Powder extracts were the material retained after original DCM/methanol extraction at the MI (see chapters 3 and 4 for a full description of this). These powders were supplied in dried form and were subsequently dissolved via an alkaline extraction using 0.1 M NaOH, before final extract solutions were neutralised via HCl addition to produce a saline solution. Additionally, two powder extracts were also tested after aqueous extraction. An alkaline extraction, whilst not common, was chosen based on similar work which showed it to be an efficient method to ensure a high protein yield from both green tea (Shen et al. 2008) and algal sample material (Harnedy & FitzGerald 2013; Harnedy & FitzGerald 2013). A full methodology concerning the extraction method, from powder production to their dissolution via alkaline or aqueous extractions can be found in chapter 4.

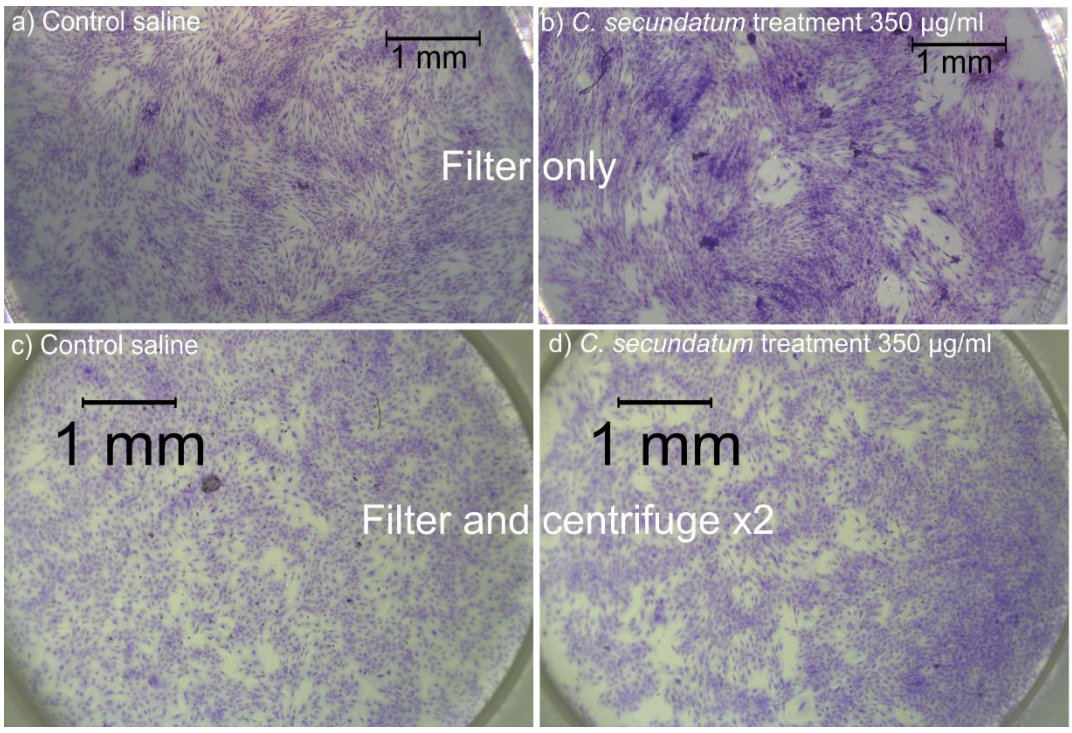


Figure 2.7: collage showing day 7 hBMSCs [a) b) donor: 001, passage:4; c) d) donor: 002, passage:5] challenged with either a control (saline 10%) or *C. secundatum* 350 µg/ml treatment. *C. secundatum* treatment of b) was filtered after being added to culture media, whilst d) was also centrifuged twice before media addition. Images show the whole of one treatment well, from a 96 well plate, stained with CV.

Whilst powder extracts proved to be a very interesting source of bioactivity, one issue of undissolved residue material remaining in cell culture media did require optimisation during

the project. This residue became incorporated into cell monolayers where it was subsequently stained during CV assays (see section 2.5.2 for further detail on this assay), giving false positive cell proliferation values. To combat this, extracts were centrifuged when tested during hFOB screening of chapter 3, or filtered (0.22 µM, results not shown). Both of these methods reduced the amount of residue material that was present but did not completely eliminate it. Instead, a combination of two cycles of extract centrifugation and filtration (0.22 µM) of cell treatment media prior to use was required to eliminate residue. Differences between the two methods can be seen in figure 2.7, whereby *C. secundatum* treatment which was filtered and centrifuged had much less associated stained residue than that prepared with filtration only.

2.5 Choosing an accurate, reliable method of measuring cell proliferation

2.5.1 PicoGreen

PicoGreen reagent is a highly sensitive nucleic acid stain, which is reported to detect much lower DNA quantities than other commonly used assays, such as Hoechst 33258. This assay is also stated to benefit from a higher fluorescence level than other traditional kits, giving less signal contribution from RNA and single stranded DNA contaminants. It is an established technique, having been in use for almost two decades (Singer et al. 1997), which along with its reported benefits (e.g. high sensitivity) made it a promising choice for initial work.

Early PicoGreen results were used for preliminary tests, such as choice of cell seeding density (see section 2.3.3), and for determining proliferative effects of 43 marine extracts dissolved in DMSO (see chapter 3). These tests were informative, but also collectively highlighted several issues with this assay. Firstly, the standard curve used for converting fluorescence values to DNA concentration has a fairly limited range, with a tendency for reporting error values if cell seeding density or growth is too high. Extrapolation beyond the standard curve can be used to calculate DNA concentration, though a linear relationship cannot and should not be assumed. Ideally, test solutions should be diluted and a second test performed, but this then involves extra pipetting steps (one for diluents and one for cell solution) increasing error, as well as necessitating multiplication of values. Standard dilutions can be made based on time point length (i.e. a greater dilution with longer culture time), which generally works well for cell lines. However, primary cell growth shows significant patient to patient variability, meaning dilutions must be determined for each batch used. Ng *et al.* (2005) also highlighted concerns surrounding establishing linearity and suitable dilutions, as well as

issues with DNA as a measure of cell proliferation. For example, DNA concentration varies with the cell development cycle, meaning it doesn't give a constant expression level and therefore is only an approximation of cell proliferation; though arguably this is a problem with most assays.

PicoGreen samples also require numerous processing steps, including a 2x PBS/buffer wash, addition of lysis solution, mixing prior to freezing, at least one freeze thaw cycle, mixing post freezing and finally plating out of test solution, which often includes a dilution step. This is time consuming and increases the risk of variation in test volumes between wells, both through pipetting error and evaporation. Including duplicate samples also means multiple plates must be used, making plate to plate variability a factor (though standard curve solutions are included on each plate to limit this). In addition to these practical concerns, PicoGreen is also a relatively expensive technique, due mainly to reagent cost. These reagents also degrade over time and in exposure to light, necessitating comparisons between different reagents and their eventual replacement. Table 2.2 highlights this reagent variability, with a difference of 779 relative fluorescence units between reagent 2 and 3 at the 1000 ng/ml concentration.

Table 2.2: relative fluorescence units detected by three different PicoGreen reagents over the suggested standard curve range. Each reagent was exposed to the same solutions containing λ DNA standard at the specified concentrations. Values were detected at excitation wavelength 480 and emission 520nm (n=2).

	Relative Fluorescence Units (Ex: 480nm, Em: 520nm)		
λ DNA standard concentration (ng/ml)	Reagent **1**	Reagent **2**	Reagent **3**
1000	4772	5139	4360
100	757	748	675
10	164	174	152
1	105	98	100
0	86	97	86
R-squared value from standard curve:	0.9983	0.9992	0.9987

One useful example of PicoGreen reliability is a study which measured the proliferation of meniscal fibrochondrocytes on different microcarriers, as it compared DNA quantification to MTT and MTS colorimetric assays (Pabbruwe et al. 2005). DNA level did not allow for differentiation between the two lowest cell seeding densities, a trend also seen to some degree in this work (figure 2.2), whilst in comparison MTS and MTT were much more sensitive. Both colorimetric techniques also detected differences in proliferation between microcarriers, whilst PicoGreen showed no variation at any cell density. Again, a similar trend

was seen in this work, whereby only small differences in proliferation were seen between the DMSO based treatments of chapter 3. This could be a real reflection of extract effects, but may also be influenced by PicoGreen as an assay choice.

Overall, it seems PicoGreen is capable of measuring of cell proliferation, but has several hindrances which make it an impractical assay for large scale screening. An ideal proliferation measure would be simple, quick, inexpensive, involve limited processing and pipetting steps and be capable of detecting clear differences between treatments, with good inter and intra variability. MTT and MTS may seem obvious alternatives and are popular choices within the literature, though are not actually measures of cell proliferation. Instead they measure cell viability, via metabolic activity, and should not be reported as proliferation measures.

2.5.2 Crystal violet

CV assay was selected as a potential replacement for PicoGreen as it is a true proliferation measure which appears to address many of the latter's shortcomings. CV utilizes the triphenymethane dye (4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline), which has a wide variety of different applications (Vega-Avila & Pugsley 2011). Amongst these is determining cell proliferation, as the dye stains DNA present in cell monolayers. After solubilisation, the colour intensity is quantifiable through an absorbance reading which is proportional to cell number. This assay has obvious benefits; it is simple to undertake, cheaper and faster than other absorbance viability measures such as MTT, which requires a 4-hour incubation period. Furthermore, initial preparation of test plates, including washing, fixing and staining, are all completed to saturation by covering the entire monolayer in excess solution. Pipetting error is therefore limited to a 100µl addition of solubilisation solution, which may in part explain CV's low intra (figure 2.9) and inter (see later results) variability.

One of CVs first reported uses was as an approximation of cell death, through measurement of residual cell number, a technique which was shown to be more sensitive than others available at the time (Flick & Gifford 1984). Subsequently, this method was adapted to stain cell monolayers grown on 24 well plates (Gillies et al. 1986), before finally being optimised for 96 wells in a method similar to that used in this work (Kueng et al. 1989). It is therefore a much older method than PicoGreen, but is well established within the literature and still frequently used. For example, CV was used to measure the proliferative effects of fucoidan (Kim et al. 2015), as well as cell migration stimulated by nacre (Lee et al. 2012). It is also often used in conjunction with other assays to measure cell attachment on different scaffolds (Li

et al. 2015), such as on glycosaminoglycans (Salbach et al. 2012) and titanium surfaces (Bezerra et al. 2017).

Direct comparisons of CV results with DNA and other cell proliferation measurements are not common in the literature, though one study investigating breast cancer cells did briefly compare different techniques (Zivadinovic et al. 2005). These results weren't presented, but CV was reported to correlate very well to both DNA content measurements and haemocytometer cell counts. Furthermore, MTT assay results were displayed and showed a linear correlation with those of CV. Also of note, CV standard error bars were very small in this study, supporting the previously stated low variability of this assay. Another study investigating breast cancer cell growth also included data from multiple assays; including CV, PicoGreen, MTT and Hoescht 33258. All were comparable in their measurement of cell response, showing significant increases in proliferation after Interleukin-7 administration (Al-Rawi et al. 2004).

However, this technique is not without its own limitations. Firstly, whilst cheap all required test solutions are highly toxic. Cell seeding density is again an important factor, though this time to ensure monolayer stability rather than detectable fluorescence levels. For example, if seeding density is too low, potential differences between treatments are difficult to observe. Alternatively, if too high monolayers tend to become unstable, forming a double or triple layer of cells which may fold over during culturing (particularly at longer timepoints), causing them to be washed away prior to fixing. Pipetting may also scratch monolayers and remove cells if not performed carefully (figure 2.8), an early problem which was remedied in later assays by rapidly inverting test plates to remove excess solution.

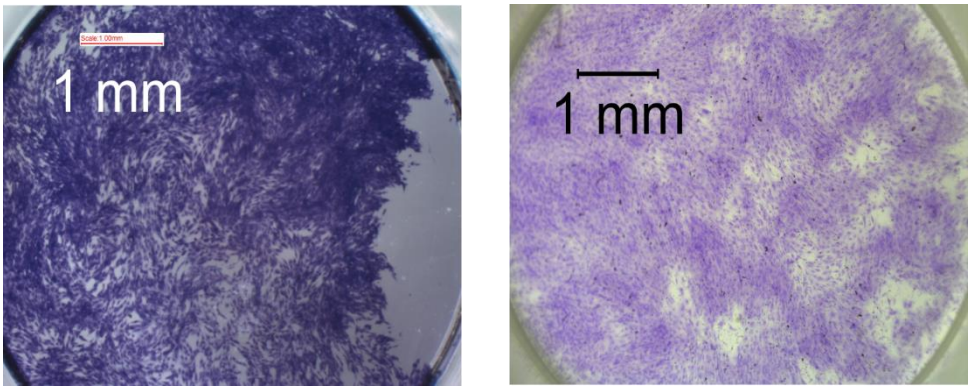


Figure 2.8: two images of a single well from a 96 well plate, stained with CV. The image on the left shows a confluent layer of cells washed by pipette multiple times. The right image shows a confluent layer washed via flicking.

Finally, it is important to note that CV stain is not specific to cells and will also stain other material present within test wells, such as extract residue (as discussed in section 2.4.2). However, assuming seeding density is optimised and residue limited, this is a useful cell proliferation method; which in this work was consistently more reliable than PicoGreen.

2.5.2.1 Crystal violet – optimisation

Use of CV necessitated another seeding density optimisation test, to ensure a density was chosen that would allow trends to be identified over the full 7-day test period. Figure 2.8 shows that $1x10^5$ and $5x10^4$ cells/cm^2 densities were too high, quickly reaching confluence and becoming indistinguishable by day 4. $1x10^3$ cells/cm^2 was too low and showed little change over time, though $1x10^4$ cells/cm^2 was again optimal, showing clear differences between time points. Variability was also excellent, with each mean value having tight standard deviation, particularly in comparison to PicoGreen results (see section 2.3.3).

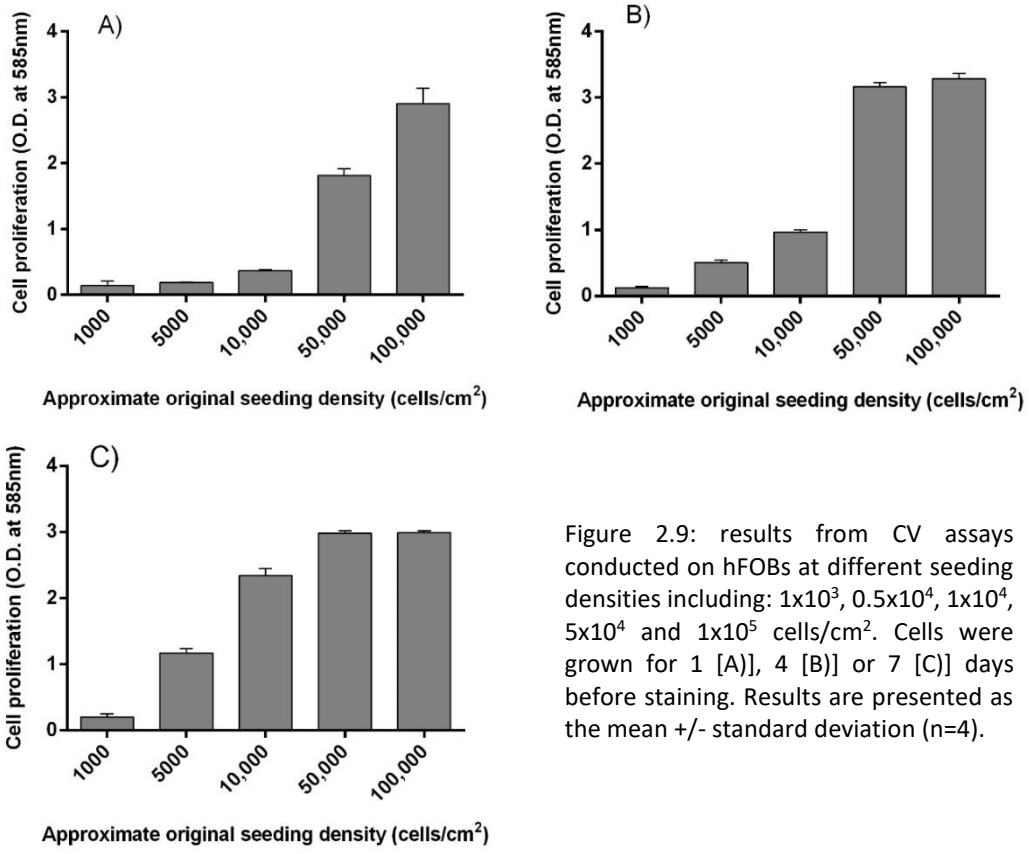


Figure 2.9: results from CV assays conducted on hFOBs at different seeding densities including: $1x10^3$, $0.5x10^4$, $1x10^4$, $5x10^4$ and $1x10^5$ cells/cm^2. Cells were grown for 1 [A)], 4 [B)] or 7 [C)] days before staining. Results are presented as the mean +/- standard deviation (n=4).

Also of note from figure 2.9, is that increases in optical density values showed a very linear relationship with seeding density. This is to be expected, as more cellular material is available

to stain, but also suggests that optical density is a good representation of cell number. Figure 2.10 is a continuation of this, whereby both hFOBs and hBMSCs (from later experiments – see section 2.6 for explanation of this term) were seeded in a 2x dilution gradient, with cell number per well ranging from 130 to approximately 16,667. As figure 2.10 shows, the relationship between optical density and cell number is very linear, with an R^2 value of 0.9993. Not only does this validate CV as a measure of cell proliferation, but it also allows for optical density values to be converted to cell number.

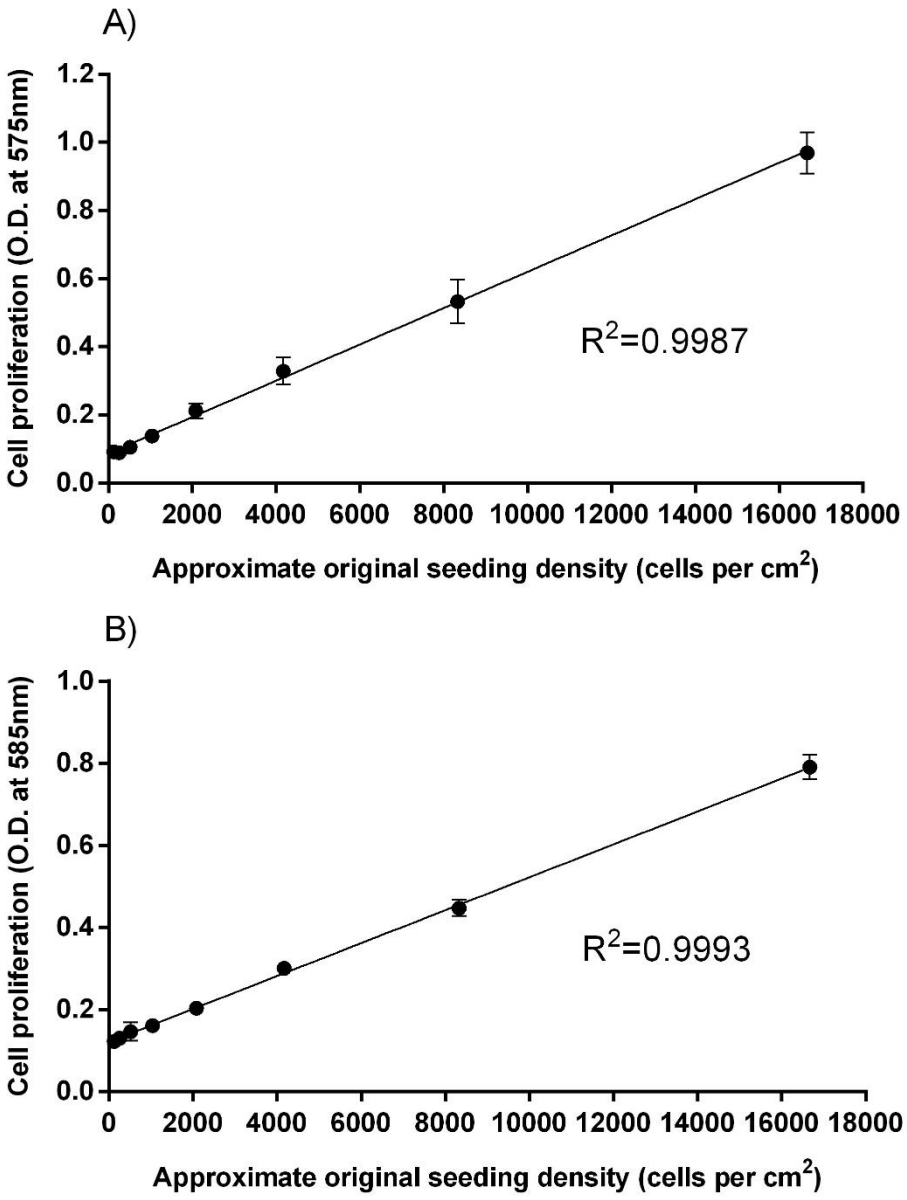


Figure 2.10: results from a CV assay performed on hFOBs [A)] and hBMSCs [B)] (donor 002, passage 5). hFOBs and hBMSCs were seeded at densities ranging from approximately 130 to 16,667 cells/well in a 2x dilution gradient. Cells were given a 24-hr attachment period and then immediately stained afterwards, limiting proliferation time and therefore variation from the original seeding density. Results are presented as the mean +/- standard deviation (n=6).

2.6 Primary cells (hBMSCs)

2.6.1 Defining the cellular component of skeletal regeneration

Stem cells are often defined in terms of two key traits; namely a self-renewing capacity and an ability to differentiate into cells from multiple lineages (Morrison et al. 1997; Reya et al. 2001). It is these characteristics, amongst others, that make stem cells so promising for clinical applications, fuelling research in this area (Morrison et al. 1997). Much interest in this field was initially focused around pluripotent embryonic stem cells, first cultured by Evans & Kaufman (1981), due to their potential for generating all types of human tissue. However, these cells were plagued by ethical and immunological concerns (Brooks 2012), whilst even immunologically compatible induced pluripotent stem cells present issues for clinical use, such as insertional mutagenesis (Teo & Vallier 2010). Consequently, other stem cell sources have been intensely researched since this early work. These include, amongst others, circulating stem cells associated with mammalian blood (Kuznetsov et al. 2001); umbilical cord blood multilineage cells, which show long term viability as a cell reserve (Eom et al. 2014); placental derived mesenchymal stem cells, which, similar to bone marrow derived cells, have a multilineage differentiation potential (Miao et al. 2006); and adipose tissue-derived stem cells. The wealth of sources and associated published material can result in confusion as to what constitutes a stem cell, facilitating more generalized definitions, whilst choosing the appropriate terminology is troublesome for many aspects of stem cell biology (Tajbakhsh 2009; Owen 1998).

A good example of this would be mesenchymal stem cells (MSCs), sometimes referred to as mesenchymal stromal cells or multipotent stromal cells, which have a definition that has evolved significantly over time (Keating 2012). Originally, MSCs were conceived due to an *in vivo* demonstration of the osteogenic potential of bone marrow cells by Friedenstein *et al.* (1966). Subsequent studies, including *in vitro* work (Friedenstein et al. 1970; Friedenstein et al. 1976), developed this idea and showed bone marrow derived cells to have clonal qualities and plastic adherence. Ideas on MSCs have since developed and it is now agreed that they have a multi-lineage potential, being able to differentiate into cells of adipogenic, chondrogenic and osteogenic lineages (Bronckaers et al. 2014; Hua et al. 2013; Zhu et al. 2012; Rezwan et al. 2006; Scaglione et al. 2009). However, despite intense research in this area there is still no known marker exclusive to MSCs, whilst definitions of the term are mostly limited to *in vitro*, rather than *in vivo*, demonstrations of stem cell characteristics (Keating 2012).

MSCs, preserved through their high proliferative capacity, differentiate through specific lineages to produce a wider population of mesenchymal progenitor cells (MPCs). Similarly, MSCs and MPCs can be thought of as part of a wider group of bone marrow stromal cells (BMSCs); which also contains a variety of other bone marrow support cells (Brooks 2012). However, authors may also refer to BMSCs as cells with a multilineage potential, instead of saying MSCs; or they may use the terms interchangeably. The latter appears to be most common, with the term BMSC being used in place of MSC. Therefore, to avoid confusion the term human bone marrow derived mesenchymal stromal cells will be used throughout this thesis, abbreviated to hBMSCs. This term will specifically refer to fibroblast-like cells, derived from multipotent MSCs in bone marrow, that undergo differentiation to produce bone forming osteoblasts. When MSCs are originally cultured, clonal colonies of cells with fibroblast-like morphologies are formed, referred to as colony forming unit-fibroblasts (CFU-fs; where each colony is derived from a single MSC). Such CFU-fs have a high proliferative potential and are able to differentiate down chrondrogenic, adipogenic and osteogenic lineages, depending on culture conditions (Brooks 2012; Scaglione et al. 2009; Owen 1998).

2.6.2 Rationale of use

Primary cells were chosen to be used in conjunction with the hFOB cell line during this study, to give a better indication of how any treatment would affect cells *in vivo*. Whilst useful for identifying potential osteogenic activity in a quick and cost-effective manner, hFOBs are an immortalised cell line and therefore not fully representative of the "normal" osteoblast condition. One useful example of this is a study which compared the proliferation, differentiation and mineralization activities of primary cells with SaOs2, MG-63 and MC3T3-E1 lines (Czekanska et al. 2014). Each cell line showed significantly greater proliferative activity than human osteoblasts during at least 1 time point, whilst differentiation and mineralisation were also highly variable. Expression of key osteoblast genes, such as Runx2 and osteocalcin, were maintained for all cell lines, but again were often at increased or decreased levels compared to primary cells. Similar discrepancies were seen between cell line proliferation and alkaline phosphatase expression in comparison to human osteoblasts (Clover & Gowen 1994). These concerns do not mean cell lines cannot be used, however, they should not be used as a complete replacement for primary cells, and any treatment effects should be re-confirmed after initial screening.

Primary hBMSCs used in this work were acquired from human bone marrow samples, collected during vertebral screw placement at the Royal Victoria Hospital in Belfast. Whilst commercial sources of these cells are available they were not used for several reasons: 1. a limited supply per vial which means that smaller experiments would need to be devised, 2. collecting samples means inclusion and exclusion criteria can be better applied to ensure that patients were not taking any drugs that would affect results and 3. samples can be collected from many patients, allowing repeat experiments with different donors to investigate inter-patient variability.

2.6.3 Ethical considerations

Ethical approval for the collection and use of human derived tissue samples from NHS patients was granted by the North East-Tyne and Wear South Research Ethics Committee (REC ref 15/NE/0250). Informed written consent was obtained from all donors who provided samples for use in this work Inclusion criteria were those aged 18-65, who were able to consent for themselves and undergoing elective surgery requiring pedical screws; as this allowed easy access to bone marrow in the vertebral body. Exclusion criteria were those with a concomitant condition, such as rheumatoid arthritis, osteoporosis and other metabolic bone diseases, or those taking medication known to affect bone metabolism such as long term steroid use and statins. See appendix 1 for ethical consent form, appendix 2 for patient information sheet and appendix 3 for the basic information collected from patients. In total 8 patients provided bone marrow aspirate samples between March 2016 to March 2017, which all yielded viable hBMSC populations. Donor information is provided in appendix 4.

2.6.4 Culturing of hBMSCs

2.6.4.1 hBMSC extraction from bone marrow sample

Approximately 6-8 mls of bone marrow aspirate was collected by surgeons at the Royal Victoria Hospital, Belfast. Samples were mixed with 100 µl of heparin to prevent clotting and stored on ice for 1-2 hours before processing. Aspirate was first agitated using a syringe and needle, to ensure a single cell suspension. Subsequently, this solution was washed with PBS and centrifuged to remove lipid, before the cellular component was resuspended in PBS and gently layered onto 4 mls of Histopaque-1077 (Sigma-Aldrich, UK) solution. This mixture was centrifuged for 30 minutes at 500 g, to separate out the 'buffy layer' containing any white blood cells of interest. After separation, the buffy layer was isolated, washed and suspended

in a small volume of complete hBMSC media [see 2.6.4.2]. An aliquot of this suspension was then treated with 2% acetic acid to remove red blood cells, before being counted using a Neubauer haemocytometer. Cells were subsequently added to T-75s at a density of 1-3x10^5 cells/cm^2, left for 7 days undisturbed and subsequently fed twice weekly until confluent.

2.6.4.2 Flask, plate culturing and cryopreservation of hBMSCs

For normal cell growth, cells were cultured in 'complete media' which consisted of α-MEM media (22561-021, ThermoFisher Scientific, UK) containing 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin-streptomycin (pen-strep) (15140, Gibco, UK). Cells were cultured in a humidified incubator at 37°C and 5% CO_2. In experiments which required cell differentiation this media was supplemented with 50µg/ml ascorbate-2-phoshpate (A8960, Sigma-Aldrich, UK), 2 M β-glycerophosphate (9422, Sigma-Aldrich, UK) and 0.01 µm dexamethasone (D2915, Sigma-Aldrich, UK)- termed 'osteogenic media'.

For experiments, hBMSCs were cultured at a seeding density of 2x10^4 cells/cm^2 and at no later than passage 6 to help ensure phenotype stability. Cryopreservation follows the method of 2.3.2.3, except α-MEM media was used instead of DMEM/HAM F12.

2.6.5 hBMSC proliferation optimisation

As with hFOBs, establishing an optimal seeding density was key before starting large scale experiments with hBMSCs. However, at this stage in lab work difficulties surrounding correct solvent choice and concentration had also become apparent. Therefore, an experiment was carried out to assess cell growth at three different densities using both DMSO and ethanol. 0.1, 0.5 and 1% ethanol were used, along with 0.1% DMSO – as DMSO had already proven to have cytotoxic effects at higher concentrations. Figure 2.11 shows day 1, 4 and 7 data for hBMSCs seeded at a 1x10^4 cells/cm^2 density. Data for control (complete media), DMSO and ethanol treatments are displayed at each time point, and for the most part cause only minor changes in cell proliferation. Day 1 data is the most variable, though standard deviation values are still modest and are likely explained by low cell number (both due to the lower seeding density used and early day 1 time point). 1% ethanol is also increased compared to other treatments at day 1, but not to a significant degree. At day 4 and 7, all treatments were closer in value to each other, with low associated error and no significant deviations from

control – though at day 7 0.5 and 1% ethanol control – though treatments were slightly reduced in value.

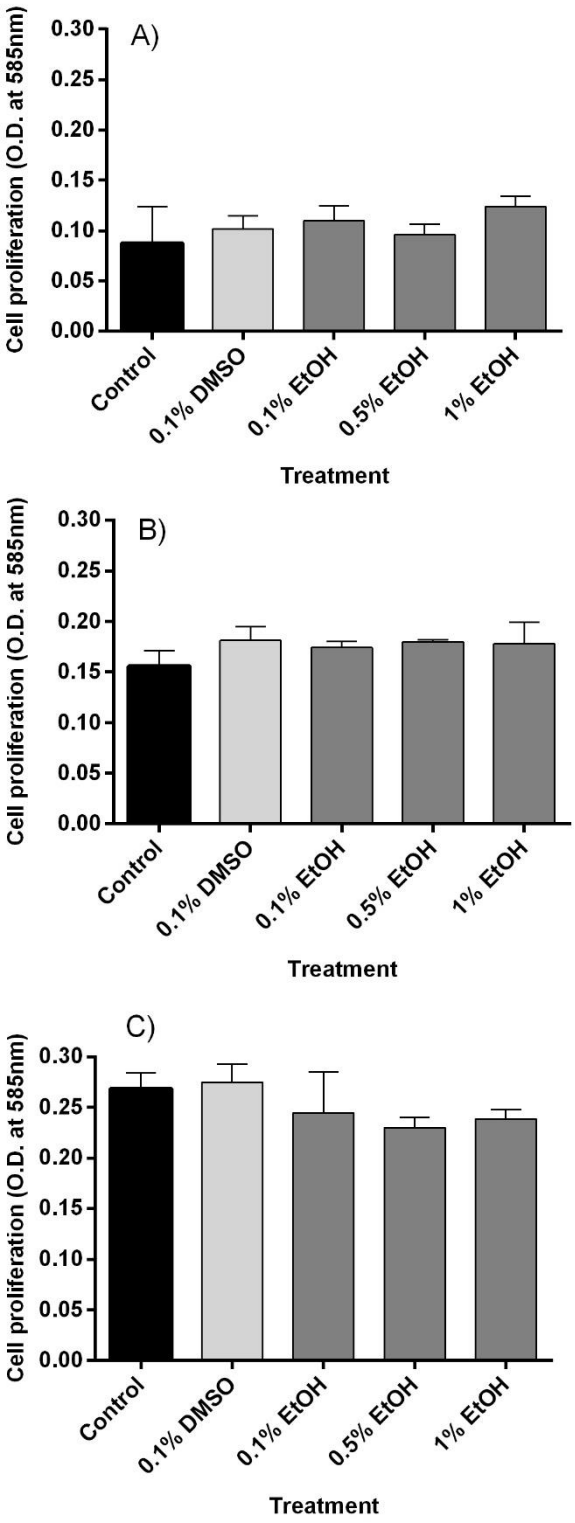


Figure 2.11: results from a CV assay performed on hBMSCs seeded at **1x10^4 cells/cm^2** at days 1 [A)], 4 [B)] and 7 [C)]. Cells were treated with either complete media (Control), DMSO (0.1%) or EtOH (0.1-1%). Results are presented as the mean +/- standard deviation (n=6 for control, n=3 for treatments).

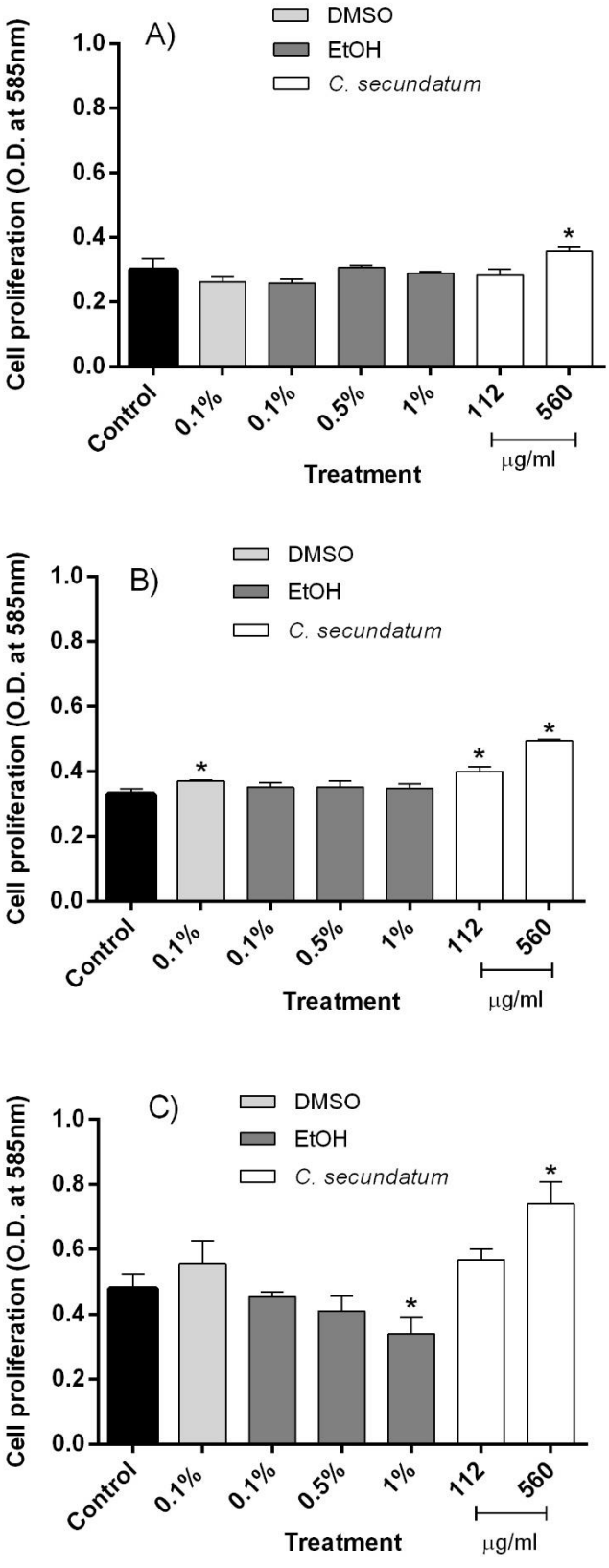


Figure 2.12: results from a CV assay performed on hBMSCs seeded at **$2x10^4$ cells/cm^2** at days 1 [A)], 4 [B)] and 7 [C)]. Cells were treated with either complete media (Control), DMSO (0.1%), EtOH (0.1-1%) or extract derived from powdered algae, denoted 614 (1 or 5%). Results are presented as the mean +/- standard deviation (n=6 for control, n=3 for treatments), *p<0.05 for treatment

As shown in figure 2.12, ethanol and DMSO again have little impact on proliferation when compared to the plain medium control, except at day 7. Here, increasing ethanol concentration decreases proliferation, leading to a significant reduction at 1%. A higher seeding density may explain the differences between figures 2.12 and 2.11 at day 7, as treatment effects are easier to discern when cells are more confluent and proliferating faster.

Finally, the highest density of $3x10^4$ cells/cm^2 (figure 2.13) maintained most of the trends seen at $2x10^4$ cells/cm^2, except at day 1 where 0.5% ethanol treatment was significantly increased relative to control. However, this is again likely a factor of the early time point, or perhaps slight variation in seeding density between wells. By day 4, treatments generally evened out in their proliferative effect, except for the slight significant increase with 1% ethanol. Day 7 is again where trends are most evident, with all treatments showing decreased proliferation relative to control, with 0.1 and 1% ethanol being significantly reduced. This is similar to previous results, except that 0.1% ethanol is significantly reduced. The reason for this is unclear, but could be due to general sources of error associated with cell culture.

Overall, this dataset shows some distinct differences to optimisation work with hFOBs. Most evident is that $2x10^4$ - rather than $1x10^4$ cells/cm^2 - is optimal for determining differences between treatments over a 7-day time course. Furthermore, hBMSCs appear to have a lag phase after plate out, as proliferation values for both day 1 and 4 time points are very similar. By day 7, cells appear to recover and proliferation increases as expected. This makes sense, as cell lines generally proliferate faster (section 2.6.2) and are less susceptible to factors such as passage number. In terms of treatment inclusion, 0.1% DMSO does not significantly decrease cell proliferation, though ethanol does have a dose dependent effect – eliminating 1% as a potential vehicle in future tests. Finally, these results also further support CV use over PicoGreen, as they are informative and have low variability.

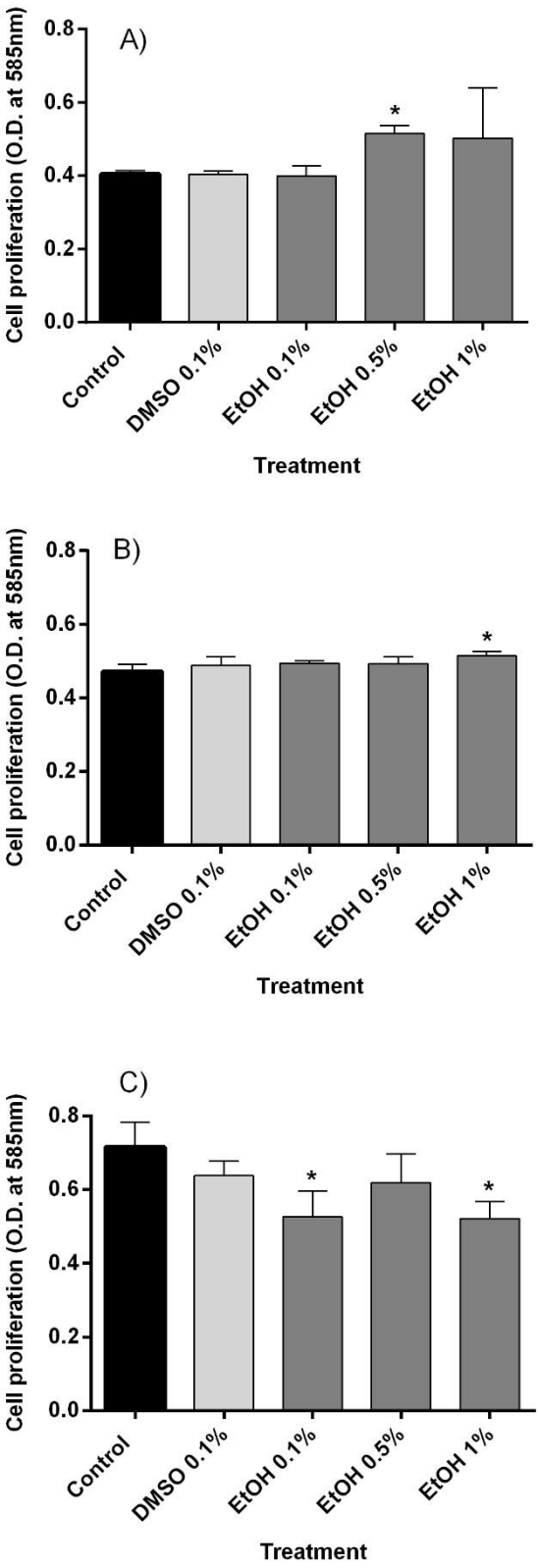


Figure 2.13: results from a CV assay performed on hBMSCs seeded at **3x10^4 cells/cm^2**, at days 1 [A)], 4 [B)] and 7 [C)]. Cells were treated with either complete media (Control), DMSO (0.1%), EtOH (0.1-1%). Results are presented as the mean +/- standard deviation (n=6 for control, n=3 for treatments), *p<0.05 for treatment compared with control

2.7 Optimisation of long term cultures

2.7.1 Alkaline phosphatase

2.7.1.1 Alkaline phosphatase – buffer and freeze/thaw issues

ALP is an early stage marker of osteogenic differentiation and was therefore a key assay when trying to detect osteogenic bioactives. However, it was also difficult to optimise for several different reasons. Firstly, any differentiation marker requires longer culturing to be expressed, as cells need to mature first. This is challenging, as healthy culture conditions need to be maintained over time for confluent test wells, which quickly produce large amounts of waste products. Changing media remedies this, though can affect monolayer stability. Even with careful feeding, cells tend to clump up over time, despite changes in FBS concentration to reduce growth rate. Once folded/clumped, cells are likely to be stressed, show greater death and produce variable quantities of ALP (or other markers).

Culturing concerns aside, a working ALP assay took a long time to establish, with early experiments detecting only very small ALP levels. One likely reason for this was that the original protocol (which is different to that of 2.2.5) was based on a pre-existing method (Palmer et al. 2015), which made use of a PBS buffer. However, other studies in this area used a variety of buffers other than PBS, including bicarbonate buffers (Almeida et al. 2001) and various tris buffers (Pathomwichaiwat et al. 2015; Bensimon-Brito et al. 2012). Another potential issue was that 3 separate freeze thaw cycles were used to ensure membrane lysis, and it was thought this could have reduced ALP activity. Therefore, an optimisation study was performed to investigate both buffer choice and freeze thaw cycle. For this, a day 7 ALP assay was conducted on hFOBs using both an alkaline (5M NaCl, 1M Tris-Cl pH 9.5, 1M $MgCl_2$) and PBS buffer, with plates exposed to room temperature or 2, 3 and 4 freeze thaw cycles. Both normal and osteogenic media were used during culturing, whilst final lysis solutions included either 0.1 or 0.2% Triton X-100.

Figure 8.1 (see appendix 5) showed clear differences in ALP expression between PBS and Tris buffers. Room temperature lysate had reduced ALP activity in PBS compared to Tris, though expression was similar between normal and osteogenic media. However, inclusion of 2, 3 or 4 freeze/thaw cycles drastically reduced ALP expression in PBS, often eliminating it. Alternatively, expression was maintained in Tris after freeze/thaw and appeared to increase with cycle number, perhaps indicating better or more complete cell lysis. Finally, 0.1 or 0.2% Triton X-100 concentration had no clear impact on ALP expression level.

This dataset answered the original questions regarding the assay, showing that both PBS buffer use and freeze/thaw cycling was reducing ALP activity. The original study which used this method, Palmer et al. (2015), tested samples at room temperature, and therefore would not have experienced a complete reduction in ALP expression. The reason behind these results is unclear, though it is likely that PBS, as a phosphate buffer, provides enough phosphate to saturate the binding site of alkaline phosphatase in cell lysate. This would then cause a significant reduction in the conversion of p-nitrophenyl phosphate to p-nitrophenyl. As testing at room temperature occurs almost immediately, ALP may not have time to be inactivated by PBS – whereas multiple freeze/thaw cycling would allow much more time for deactivation.

2.7.1.2) Alkaline phosphatase optimisation

After establishing the working assay protocol a standard optimisation test was conducted on hFOBs (figure 2.14) and hBMSCs (figure 2.15), at 7, 14 and 21 days. This aimed to ensure the assay was working on long term cultures and to establish any differences in ALP expression due to osteogenic supplementation or solvent addition.

ALP activity in figure 2.14 has been normalised to DNA content (using PicoGreen values) to account for variations in cell number between wells and treatments. In general, relative ALP activity peaked at day 7 (a), indicating early enzyme expression and cell differentiation. Furthermore, there was little variation in activity between treatments, though the highest solvent levels did cause slight decreases in activity levels, particularly for DMSO. This was likely a result of a slight cytotoxic effect from solvent addition, or suppression of cell activity. Day 14 trends (b) were slightly different, with osteogenic medium activity reduced compared to that of complete media, whilst 1% ethanol caused a slight increase in relative ALP expression. These discrepancies most likely resulted from the large associated error at this time point, which was probably PicoGreen related – as DNA values were particularly heterogenous. Of note, error is generally greater for ALP measurements than for single measures like CV due to normalisation, meaning variability in DNA readings are also a factor. However, normalisation is crucial to ensure higher ALP activity is not just a consequence of changes in proliferation between treatments, and therefore this cannot be avoided. Finally,

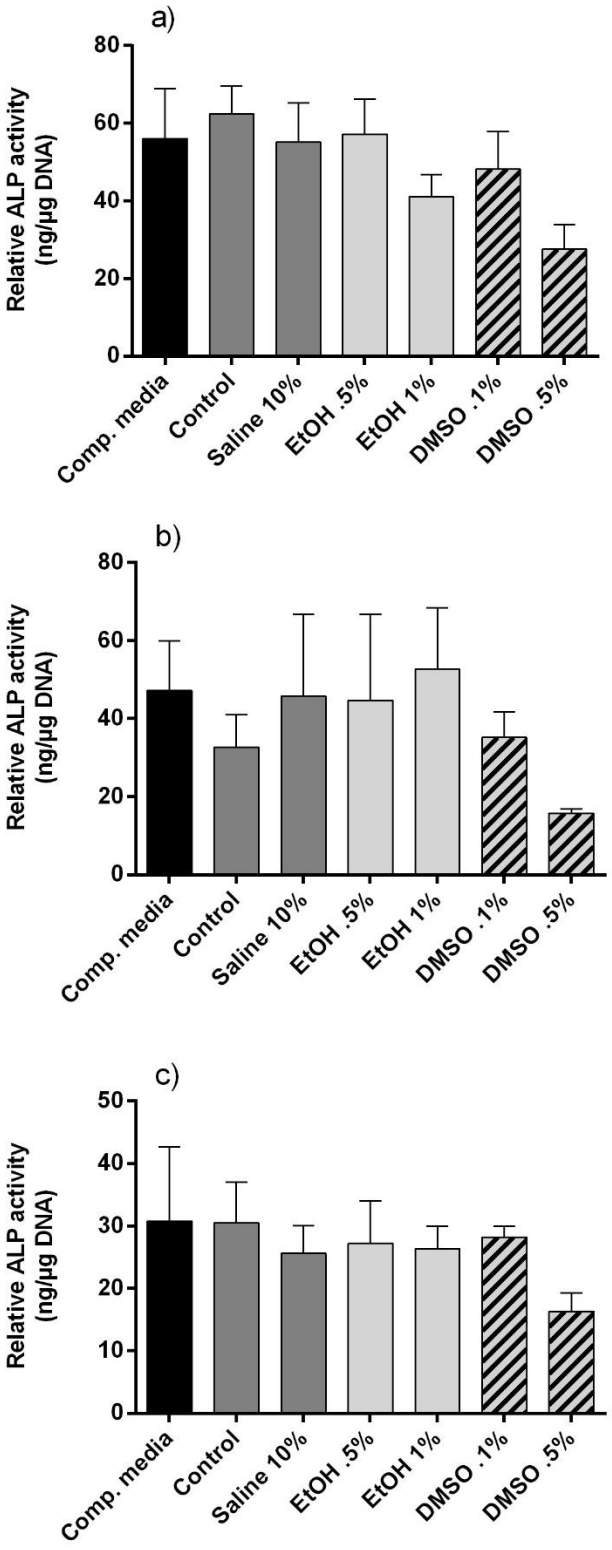


Figure 2.14: results from an alkaline phosphatase assay performed on hFOBs, at day 7 [a)], 14 [b)] and 21 [c)]. Cells were plated at a $1x10^4$ cells/cm^2 density, given 24 hours to attach and then cultured in either complete or osteogenic media. Osteogenic media also had ethanol, DMSO and saline solvents added at various percentages. Subsequently, cells were lysed via addition of a Tris buffer containing 0.2% Triton X-100 and subjected to 2 cycles of freeze thaw. Results are presented as the mean +/- standard deviation for each test condition, n=6.

it is interesting to note that complete media showed comparable ALP activity to osteogenic supplemented media. This indicates that cell differentiation was not enhanced by supplementation. Whilst numerous supplementation recipes exist for hFOBs, there are also studies which only use complete media, finding it effective (de Souza Malaspina et al. 2009) and supplier (ATCC) guidelines do not recommend supplementation. The results presented in figure 2.14 confirm that supplementation with this cell line is not necessary to promote osteogenic differentiation.

hBMSCs (figure 2.15) were much more repeatable, showing lower associated error and clearer trends. Firstly, complete media still resulted in a detectable level of ALP activity, but it was the lowest of all treatments. Osteogenic supplementation greatly increased activity levels as expected, though the inclusion of 10% saline solution in media negated this slightly. Apart from the 0.5% ethanol treatment at day 21, solvent inclusion reduced ALP activity compared to control in a dose dependent manner – though levels were still greater than those of complete media. By day 21, error increased relative to day 7 and 14, but trends were still apparent. Compared to hFOBs, primary cells displayed much greater relative ALP activity (greater than 1000 for treatments at day 21), whilst differentiation was extensively increased by supplementation. Proliferation was also lower for hBMSCs, as evidenced by PicoGreen readings and the healthy, 75% confluent monolayer evident during cultures. This is not surprising given the higher proliferative capacity of cell lines and their other limitations compared to primary cells (see section 2.3.1 and 2.6.2).

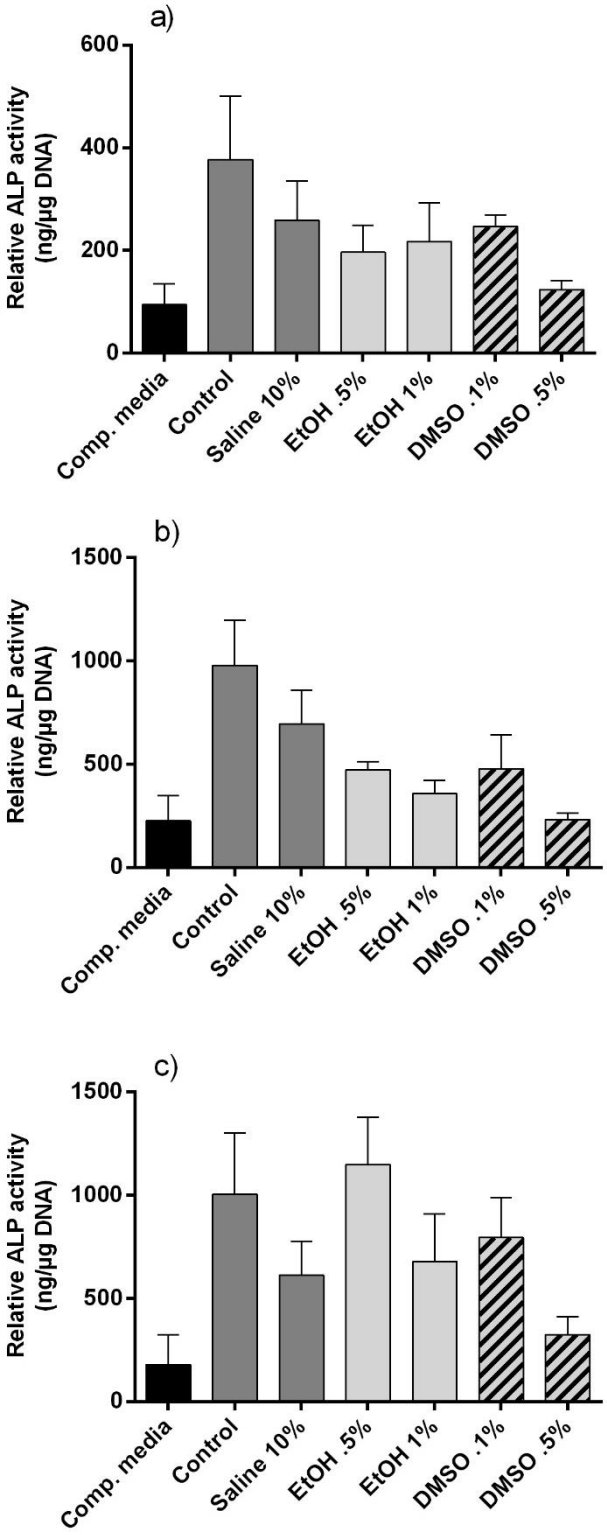


Figure 2.15: results from an alkaline phosphatase assay performed on hBMSCs, at day 7 [a)], 14 [b)] and 21 [c)]. Cells were plated at a $2x10^4$ cells/cm^2 density, given 24 hours to attach and then cultured in either complete or osteogenic media. Osteogenic media also had ethanol, DMSO and saline solvents added at various percentages. Subsequently, cells were lysed via addition of a Tris buffer containing 0.2% Triton X-100 and subjected to 2 cycles of freeze thaw. Results are presented as the mean +/- standard deviation, n=6.

2.7.2 Mineralisation optimisation

To ensure best results from alizarin red-S (see 2.2.6) staining a number of different media recipes were tested, to determine which would lead to greatest mineralisation levels of hFOBs and hBMSCs. Overall, hFOB mineralisation was more variable that that of hBMSCs (figure 2.16). For example, day 7 and 14 time points show consistently higher mineralisation levels with osteogenic media, compared to complete media control. However, day 21 osteogenic media showed reduced mineralisation, whereas at day 28 no clear trend was apparent. This variation in mineralisation may again be a result of using cell lines opposed to

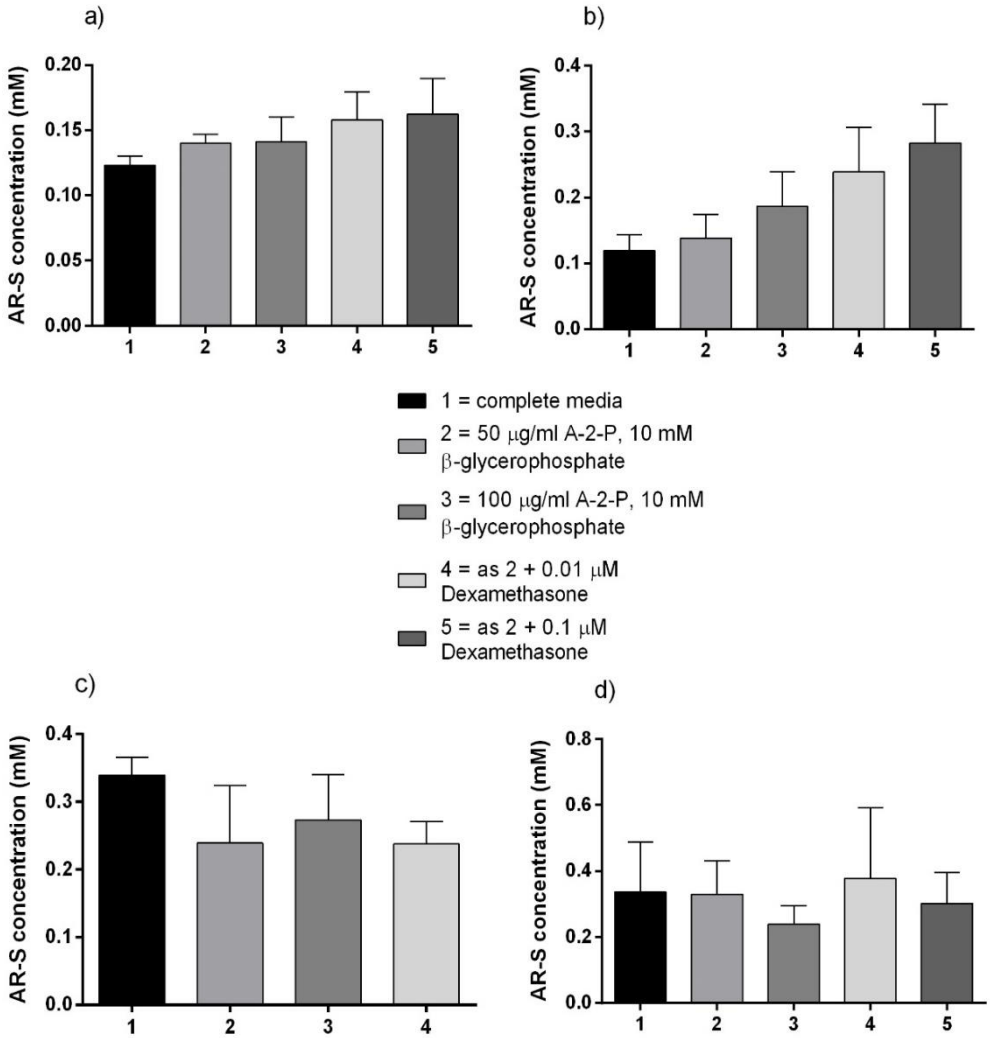


Figure 2.16: results from an alizarin red-S assay performed on hFOBs at day 7 [a)], 14 [b)], 21 [c)] and 28 [d)]. Cells were plated at a $2x10^4$ cells/cm^2 density, allowed to reach 75% confluency and then cultured in media with different concentrations of supplements, including: ascorbate-2-phosphate (A-2-P), β-glycerophosphate and dexamethasone. A complete media treatment and 4 supplemented media types were tested – see figure legend. Results are presented as the mean +/- standard deviation (n=4).

primary cells, which show more consistent patterns of mineralisation (Czekanska et al. 2014) (figure 2.17). Despite this variability, treatment 4 (50 μg/ml A-2-P, 10 Mm β-

glycerophosphate and 0.01 µM dexamethasone) produced the most frequent promotion of mineralisation (treatment 5 cells became over confluent and dis-attached at day 21 time point, explaining their absence) across all time points and was therefore chosen for future work.

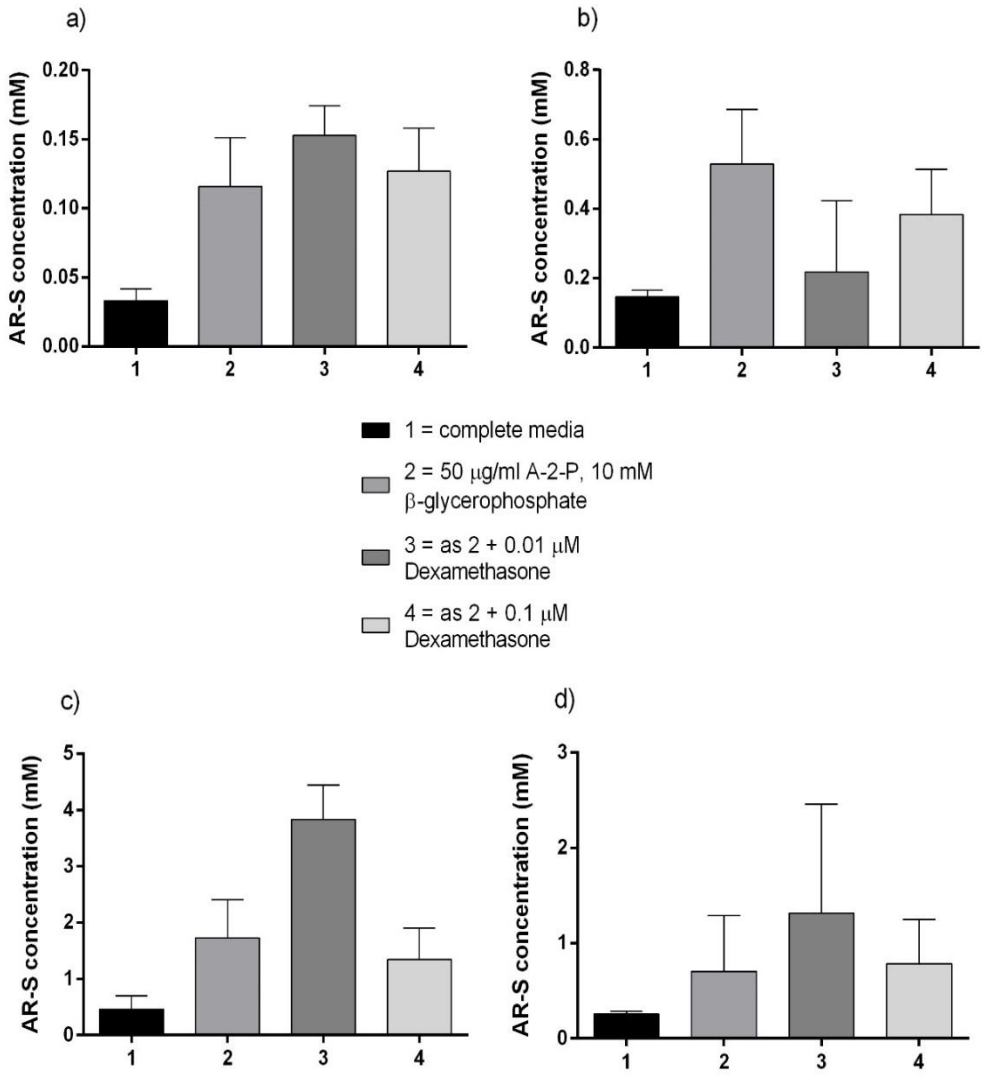


Figure 2.17: results from an alizarin red-S assay performed on hBMSCs at day 7 [a)], 14 [b)], 21 [c)] and 28 [d)] timepoints. Cells were plated at a $2x10^4$ cells/cm^2 density, allowed to reach 75% confluency and then cultured in media with difference concentrations of supplements, including: ascorbate-2-phosphate (A-2-P), β-glycerophosphate and dexamethasone. A complete media treatment and 3 supplemented media types were tested – see figure legend. Results are presented as the mean +/- standard deviation (n=4).

Comparatively, hBMSCs (figure 2.17) showed a much clearer trend than hFOBs. For example, use of osteogenic supplements promoted mineralisation level for all media recipes compared to complete media control. With the exception of variability at day 14, media recipe 3 – 50 µg/ml A-2-P, 10 mM β-glycerophosphate and 0.01 µM dexamethasone – performed best at

all time points, resulting in a large promotion of hBMSC mineralisation and was thus chosen for future work.

2.8 Summary

Overall, this chapter detailed many *in vitro* methods utilised within this thesis, why they were used and how they were tested and optimised. Throughout lab work it became apparent that assays should not be trusted solely because of their reported benefits, or wide literature use. Instead, it is important to determine how they perform between cell types/batches, experiments, time points and in relation to other similar techniques. PicoGreen was a good example of this, as whilst more modern it was not as reliable a proliferation measure as CV. Ways of improving other techniques, such as membrane preservation for CV and long-term differentiation tests, were also detailed and incorporated into assay methods. Additionally, cell culture basics were established, such as why hFOBs and hBMSCs were used and how each was cultured. It was important to test both cell types with each assay method, as well as a range of solvents. Solvents proved particularly challenging, though eventually 0.1 or 0.5% ethanol was shown to be preferable to 0.1% DMSO, whilst higher concentrations of either caused increased cell death. Furthermore, the use of alkaline extraction for powder extracts was also briefly described, including optimisation to reduce non-specific residue staining. In conclusion, method development is arguably the most important stage of any lab work, as it determines the quality of all future results. In this thesis, a limited number of *in vitro* techniques were purposefully chosen, to give specific, repeatable and reliable results on either cell death, proliferation or differentiation. This approach facilitates comparisons between the many different extracts screened, making it easier to highlight those which promote cell activity.

Chapter 3

Screening for osteogenic potential

3.1 Introduction

As of December 2017 there were approximately 242,823 accepted marine species, according to the world register of marine species (WoRMS), though this number is constantly being updated. An accurate description of marine diversity is extremely challenging and depends in part on sampling and classification effort. However, estimations indicate there may be a total of 2.2 million marine species, of which only 9% have been identified (Mora et al. 2011). This life encompasses fish; mammals; coastal, deep water and littoral invertebrates; plankton and bacteria, as well as many more taxa that do not fit within these broad groups. These organisms have adapted to survive in challenging environmental conditions, often being subjected to extremes of temperature, oxygen depletion, salinity variation and nutrient level fluctuation (Jha & Zi-rong 2004). Deep ocean hydrothermal vents are an excellent example of this, and before 1977 we were unaware of their existence. Now we understand that entire diverse ecosystems exist around these vents which are completely self-sufficient in surviving their extreme environment, requiring no external nutrient supply (Fisher et al. 2007).

In terms of the search for bioactives, high biodiversity and adaptation increase the likelihood of detecting novel molecules. Such molecules could be completely unique in structure or have functional analogues within the human body. In terms of discovering osteogenic bioactives another reason why marine organisms may be of interest is that they include many mineralising species (David W. Green et al. 2013). Depending upon the degree of conservation of molecules and mechanisms involved in mineralisation, these species may be a source of analogues that can promote activity of the BMU for bone maintenance in humans. Whilst this project focuses only on marine invertebrates, there are still a huge number of taxonomical groups to test extracts from. Positively, this increases discovery potential, but it also presents a challenge in terms of where to focus efforts. If a limited number of extracts are used tests can be more detailed, but the chances of an activity 'hit' are reduced. A trade-off is therefore necessary between the number of tests performed and extracts screened.

In relation to this project there are a wide variety of papers that make use of *in vitro* cell based assays. They can be employed to determine osteogenic activity in relatively raw, unmodified and novel substances, such as the abalone gastro-intestinal extracts mentioned in chapter 1 (Nguyen et al. 2014). Though cell viability (as determined by MTT assay) did not vary with abalone extract treatment, ALP activity showed a dose dependent increase. As ALP activity is a marker of osteoblast differentiation, and mature osteoblasts support ECM

mineralisation, mineralisation level was also quantified and showed another dose dependent increase with treatment. This demonstrates how basic assays can help to determine what aspect of cell behaviour an extract effects, in this case differentiation, justifying further tests on the mechanism of that effect. Alternatively, cell-based assays are just as useful for confirming the activity of specific molecules, such as the nacreous protein p10, that was also demonstrated to increase preosteoblast differentiation (Zhang et al. 2006). For most work in this field these basic cell activity assays are necessary for establishing an effect, and are subsequently followed by more detailed tests. Taking fucoidan as an example, there is a catalogue of work detailing the osteogenic and anti-osteoclastic activity of this sulphated polysaccharide, whilst more recent studies are now working to deduce the mechanism of these effects (Kim et al. 2014). Unlike studies on fucoidan or nacre, the extracts tested in this thesis have not been screened previously for osteogenic potential, meaning experimental work will revolve around searching for extracts which show activity 'hits'. This approach means that detail concerning bioactive structure, or the mechanism of any effects, will not be determined, yet it benefits from having a good chance of detecting extracts with novel osteogenic activity – which may show even better pre-clinical potential than that of other known marine-organism derived compounds.

This chapter will detail the first set of experiments conducted on marine extracts as part of this project. Several cell-based assays were used during the first screen, though those determining cytotoxicity and cell proliferation will be mainly focused on in this chapter.

3.2 Methods

3.2.1 Extraction procedure (for processing material, undertaken by the Marine Institute, Galway)

As previously stated, extracts used in this study came from a range of marine invertebrate taxa, collected both from shallow and deep water at numerous sites along the west coast of Ireland (see appendix 6 for list of taxa tested). Sampling work was undertaken as part of the Beaufort Marine Biodiscovery research programme, which aimed to build marine biotechnology capacity within Ireland. After collection, specimens were frozen and stored at the Marine Institute (NUI Galway) before further processing. Extraction from a specimen began with the material being freeze dried, washed with dH_2O, freeze dried again and subsequently milled into a fine powder (figure 3.1). Five grams of this powdered material was

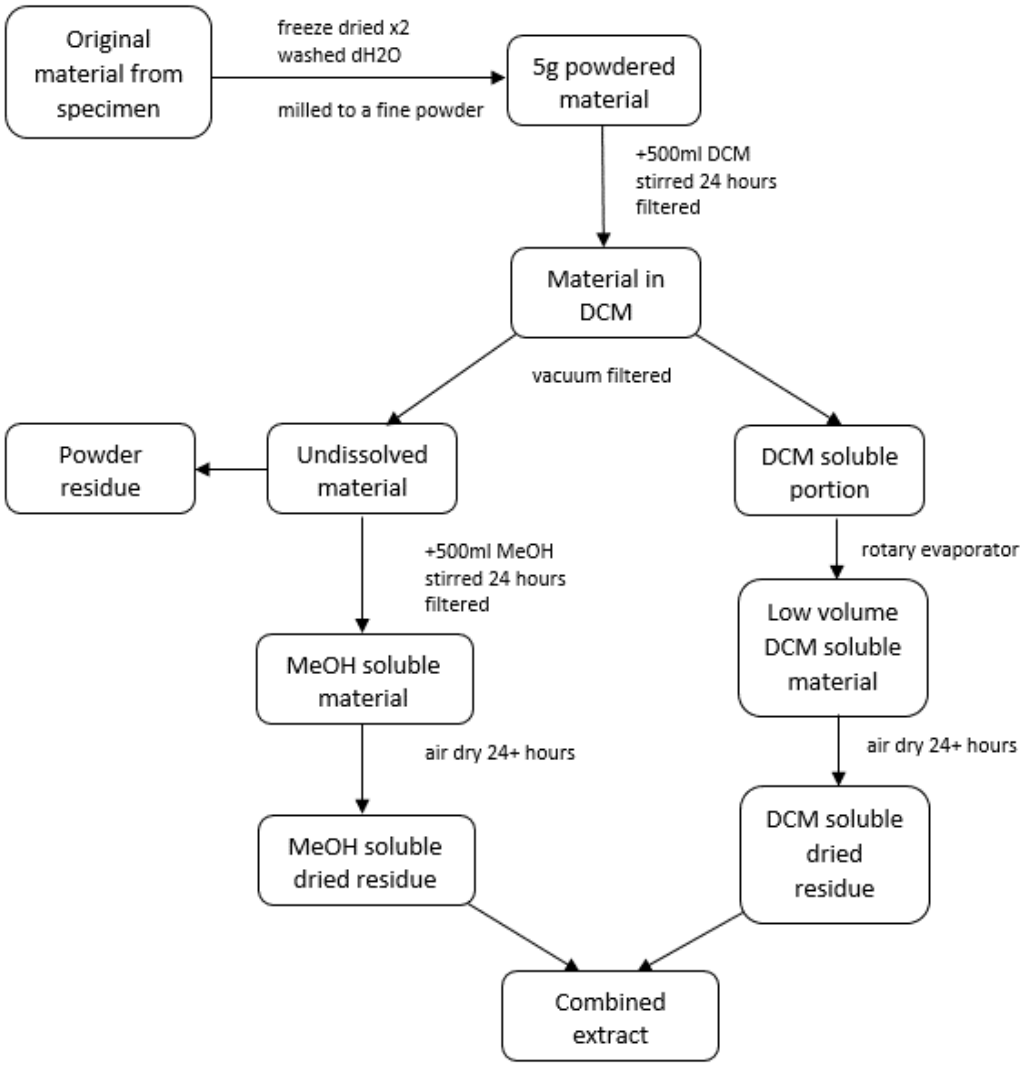


Figure 3.1: showing the steps involved in extract production, as taken by Isa Wolfe and others at the Marine Institute in NUI Galway.

then mixed with 500 ml dichloromethane (DCM, CH_2Cl_2) and stirred for 24 h at room temperature. The solution was filtered using a vacuum pump, yielding a DCM-soluble extract solution and an undissolved dried powder. A rotary evaporator was used (pressure: 685 mbar, rotation: 140 rpm [revolutions per minute], water bath temperature: ice/40°C, anti-bumping granules included) to reduce the volume of DCM to a manageable level. Any remaining solution was then placed in an open container and left for at least 24 h, producing a dried residue. The undissolved powder was then subjected to a second extraction, this time using 500 ml of methanol (MeOH, CH_3OH), using the protocol as described above (same settings as for DCM, except pressure which was set at 236 mbar). The two dried residues were finally combined, and it was this material which was shipped to QUB for use in experiments. Remaining powder (after the MeOH extraction) was collected in foil, vacuum packed and frozen for potential future use.

3.2.2 Processing material at QUB

All extracts were stored in -20°C freezers upon arrival at QUB (Queen's University Belfast) and covered in foil to prevent UV/light degradation.

3.2.2.1 Extracts MR01-MR43

Weights for this set of extracts ranged from 4 to 27.7 mg. Dimethylsulfoxide (DMSO) was used as a solvent, and in an effort to keep the concentration of extracts high a minimal amount of DMSO was added to each vial, in 100 µl increments. However, it quickly became apparent that 30 mg of material would likely dissolve in 1 ml of solvent. 100% anhydrous DMSO was also used after the first 200 µl additions, to counter the hygroscopic nature of the solvent which otherwise decreases its purity. To aid dissolution each extract vial was placed on a rotary mixer and heated repeatedly to 37°C, before finally being diluted in culture medium. When test wells were treated with 200 µl of test solution this gave an eventual concentration of extracts ranging between 4 and 6.34 µg per well, based on a maximum addition of 0.1% DMSO.

3.2.2.2 Batch of 25 dissolved in ethanol

After screening extracts dissolved in DMSO fresh material was received from the marine institute, including some pre-dissolved in DMSO and 25 in raw dried form. Of those 25 dried

extracts, ethanol was added in varying volumes depending on extract weight, to give an approximate concentration of 30 mg of material per ml of solvent. For extracts 294 and 621 the material received was minimal (0.5 mg and 1.9 mg respectively) and therefore extra solvent had to be added to ensure a manageable amount of solution to work with. Each vial was then vortexed, heated to 37°C on a rotary shaker and placed in a low-frequency sonicator (Branson 3510, USA). For proliferation studies using hFOBs a maximum ethanol concentration of 0.5 or 1% was used, which resulted in 30 or 60 μg (respectively) of extract material per 200 μl test solution (except for 294: 8.32 or 4.16 μg per 200 μl, and 621: 34.4 or 17.2 μg/200 μl).

3.2.2.3 Powder residues

Powder residues are the material which did not dissolve in either DCM or methanol during the original extraction process. For each powder extract 60 mg of powdered material was added to a 20 ml universal. A minimum addition of 2 ml of solvent was required to reconstitute the powders. For most powders 2 ml of 0.1 M NaOH was added to each universal, which was subsequently subjected to cycles of vortexing, placement on a rotary shaker, heating to 37°C and placement in a low-frequency sonicator. To exclude undissolved material from treatments each solution was either centrifuged and the supernatant collected, or filter sterilised using a 0.22 μm filter. A small volume of 2 M HCl was then added to neutralise the solution (monitored via pH meter), giving a 100 mM solution of NaCl, containing dissolved extract material. To make final treatment solutions these solvent extracts were mixed with culture medium at either 0.1, 0.5, 1, 5 or 10% concentrations.

For the powder residue 621, three other solvents were also tested, to determine the effect of solvent choice on treatment solution bioactivity: 70% ethanol, 0.1 M HCl (then neutralised with NaOH) and pure distilled water. Other than the solvent choice these solutions were then treated in the same method as described for the NaOH extraction. 621 was chosen at random, as use of three solvents on all powders would have resulted in an unmanageable number of treatments.

3.2.3 Cell culture and activity assays

hFOBs were cultured as detailed in chapter 2 (section 2.2).

Cell based activity assays included:

- XTT cell viability – see section 2.2.1.
- Lactate dehydrogenase cytotoxicity – see section 2.2.2.
- PicoGreen cell proliferation – see section 2.2.3.
- Crystal violet cell proliferation – see section 2.2.4.
- Alkaline phosphatase cell differentiation – see section 2.2.5.

3.2.4 Experimental design

The seeding density used, unless otherwise stated, was $1x10^4$ cells/cm^2 for the experiments detailed in this section. Cells were also given a 24 hour 'attachment' period, after which point the culture medium was removed and replaced with that containing treatments. All medium additions, normal or treatment, were 200 µl in volume. During culturing, observations were noted on set days.

3.2.4.1 Extracts dissolved in DMSO MR01-43

- For proliferation/LDH testing extracts were seeded into 96 well plates, with 4 repeat wells per treatment. Cells were fed on day 4 with treatment medium.
- During culturing two controls were included: **1.** complete medium only and **2.** complete medium+0.1% DMSO.
- XTT was conducted after 1 day of treatment.
- Cytotoxicity at day 1 was measured using LDH assay
- PicoGreen assays were performed at day 4 and 7 on cell lysates (after lysis using 0.1% Triton X-100 and four cycles of freeze and thaw) with a set of standards on each plate for standard curve calculations (accounting for plate to plate variability). For both LDH and PicoGreen tests, supernatant was tested in duplicate—meaning that for each well cultured two values were obtained, giving a total of 8 values per treatment.
- For ALP activity cells were cultured in 96 well plates with four repeat wells per treatment. hBMSCs (donor: 008, passage: 4) were used instead of hFOBs and cells were fed on day 4. Controls included: **1.** complete medium only and **2.** osteogenic medium+0.1% DMSO. ALP readings were normalised to DNA concentration, using PicoGreen assay, and supernatant was tested in duplicate.

3.2.4.2 Batch of 25 extracts dissolved in ethanol

- Cells were plated in 96 well plates with 4 repeat wells per treatment.

- During culturing a total of three controls were included: **1.** complete medium only, **2.** complete medium+0.5% EtOH and **3.** complete medium+1% EtOH.
- Cytotoxicity at day 1 was measured using LDH assay
- Proliferation was measured by crystal violet staining at day 4 and 7 (fed day 4). Unfortunately, the day 1 cell monolayer detached during washing and this crystal violet time point was lost.
- For differentiation testing, cells were seeded onto 96 well plates for ALP activity assay with four repeat wells per treatment. hBMSCs (donor: 008, passage: 4) were used and cells were fed on day 4. Controls included: **1.** complete medium only and **2.** osteogenic medium+0.5% EtOH. ALP readings were normalised to DNA concentration, using PicoGreen assay, and supernatant was tested in duplicate.

3.2.4.3 Powder residues

- Powder residue extracts were tested on cells seeded onto 96 well plates with three repeat wells per treatment.
- For each powder, five concentrations of 0.1, 0.5, 1, 5 and 10% were included.
- Two controls included: **1.** complete medium and **2.** complete medium with either 0.1, 0.5, 1, 5 or 10% NaCl.
- For extract 621 different solvents were also investigated [as detailed in section 3.2.2.3] and appropriate vehicle controls were therefore included.
- Cytotoxicity was determined by LDH assay at day 4
- Crystal violet assays were performed for cell proliferation on days 1, 4 and 7.

3.2.5 Statistical analysis

Results are presented as means +/- standard deviation, based on at least 3 single repeat values for each data point. Each dataset was tested for normality using both Kolmogorov-Smirnov and Shapiro-Wilk tests, as well as equal variance using Brown-Forsythe test. Subsequently, sets were tested by One-Way ANOVA, with any significant differences further investigated by Dunnetts t-test (two-sided). This compared each treatment to the medium and vehicle control group. Only two datasets did not meet the assumptions required for parametric testing and for these the Kruskal-Wallis test was used as an alternative. This included results shown in figures 3.9 b) and 3.10 b). All statistics were produced using SPSS 19 (IBM).

3.3 Results

3.3.1 Extracts MR01-MR43

3.3.1.1 XTT assay

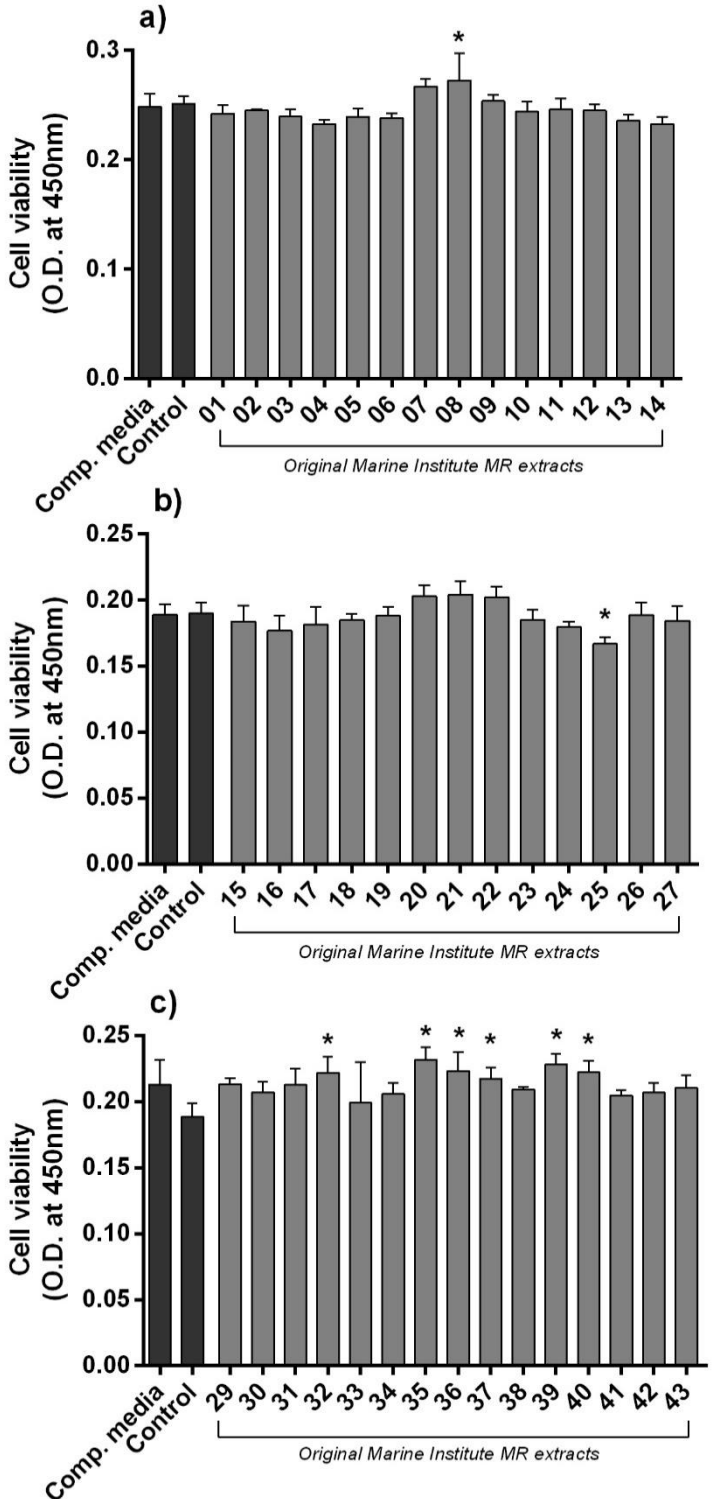
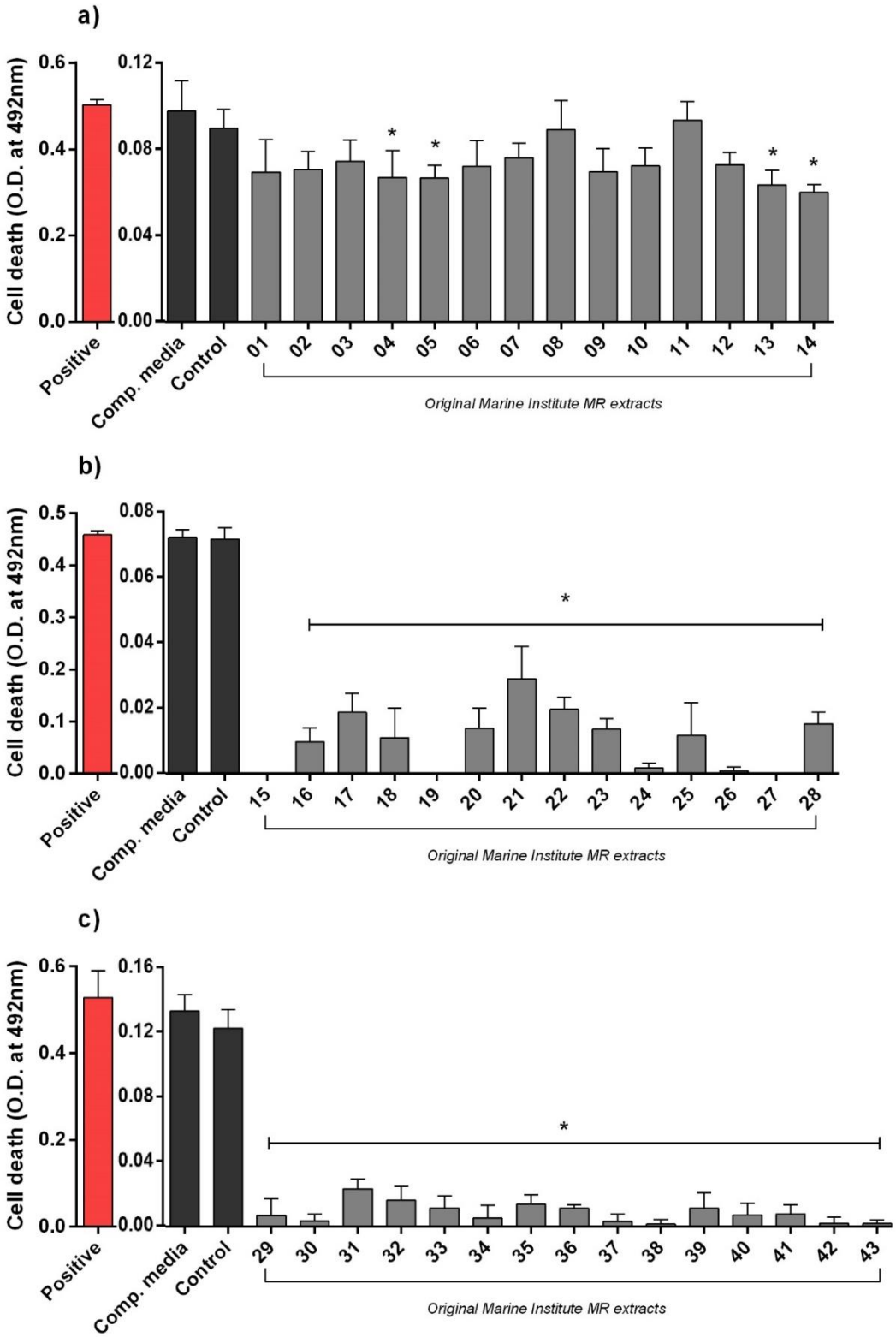


Figure 3.2: cell viability (XTT assay) of hFOBs using extracts **a)** MR01-14, **b)** MR15-28 and **c)** MR29-43. Cells were given a 24 h attachment period, 24 h with treatment and 4 h with treatment and the reaction substrate. Results are presented as the mean +/- standard deviation (n=4). *p<0.05 for treatment compared with the control (complete media + 0.1% DMSO).

Within set 1 [figure 3.2 a)] the majority of extracts only show small variations from control values. Of these MR07 and 08 are most pronounced, both showing an increase from the control. MR08's increase is statistically significant ($p=0.023$), though this treatment also shows comparatively large standard deviation values. Values for set 2 [figure 3.2 b)] extract treatments (MR15-28) vary little from the control. Within these values those for MR21, 22 and 23 are increased compared to the control, whilst MR25 and 26 are lower. However, of these the decrease in absorbance for MR26 is the only significant difference ($p=0.048$). Set 3 [figure 3.2 c)] treatment values are all increased relative to the 0.1% DMSO control. Of these increases, 6 are statistically significant ($p<0.05$), including MR32, 35, 36, 37, 39 and 40, meaning there are more metabolically active cells in these wells.

3.3.1.2 LDH assay



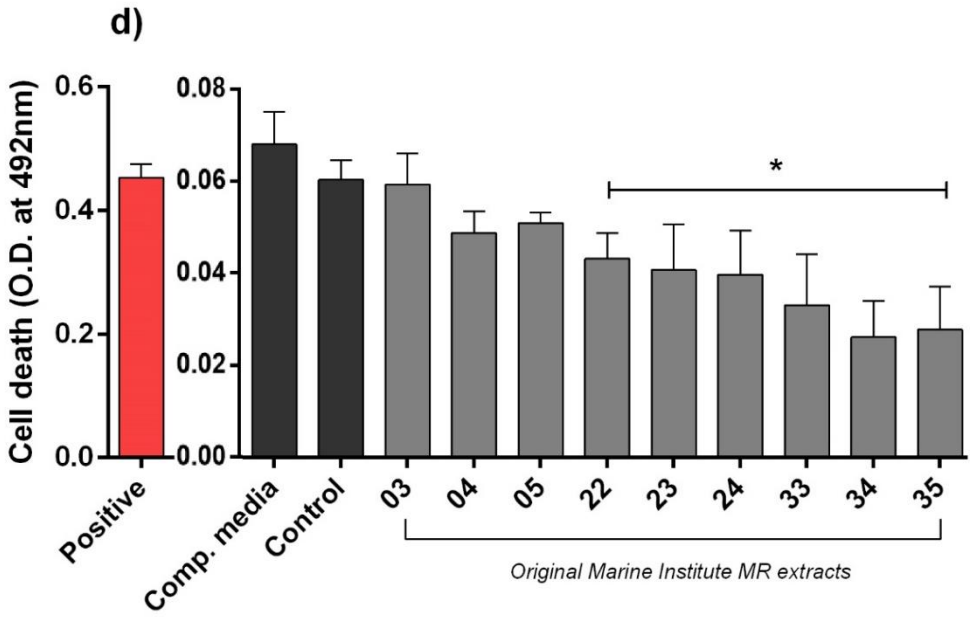


Figure 3.3: cytotoxicity results (LDH assay) for hFOBs challenged with extracts **a)** MR01-14, **b)** MR15-28, **c)** MR29-43 and **d)** 9 extracts, 3 from each identified set of extracts (see text). Cells were given a 24 h attachment period, 24 h with treatment and then supernatant was collected for testing. Results are presented as the mean +/- standard deviation (n=4). Positive shows the absorbance values for 100% cell death. All values are corrected for background absorbance by subtracting appropriate negative control values. Kit positive control confirmed the assay worked for each test. *p<0.05 for treatment compared with control (complete media + 0.1% DMSO).

Results across the three separate LDH test sets [figure 2a-c)] are comparable in many ways. The reliability of each assay was confirmed by a positive kit control. Additionally, 100% cell-death positive controls (red bar) had similar absorbance values between sets, though were slightly higher for set 1 (approximately 0.6, compared to 0.5 for set 2 and 3). All treatment data is also within a similar maximum and minimum range, with absorbance values spread between 0 and 0.16.

The main difference is seen for set 1 [figure 3.3 a)], where values for complete media control (Comp. media) and control (0.1% DMSO) are closer to those of treatment groups. This is different to sets 2 and 3 [figure 3.3 b), 3.3 c)] where cytotoxicity is markedly reduced for all treatments (generally below 0.02) compared to controls (at around 0.12). Within set 1, extracts MR04, 05, 13 and 14 are significantly lower in value compared to the control ($p<0.05$). Conversely, extracts MR08 and 11 are on a level with controls, whilst all other treatments show small cytotoxicity reductions that lack significance.

Set 2 [figure 3.3 b)] treatment cytotoxicity values (MR15-28) are all significantly lower ($p<0.05$) than those of the control. Of these, MR15, 19, 20 and 24 are the lowest, and are very close to absorbance values of zero. Set 3 [figure 3.3 c)] is similar, in that all extracts

(MR29-43) have significantly reduced ($p<0.05$) toxicity levels compared to the control. Within these, the most pronounced reductions are seen for MR30, 37, 38 and 42.

To determine repeatability, 3 extracts from each set were retested [figure 3.3 d)]. Within this test, set 1 extract values (MR-03, 04 and 05) are closest to those of the control, with extracts from set 2 and 3 showing comparatively lower absorbance levels. Comparing between the retest and original toxicity assays, set 1 values [d)] are within a similar range to those of original results [a)], though MR04 and 05 are no long significantly reduced. Sets 2 and 3 on the other hand retain their significant reductions in cell death compared to control [d)], though it should be noted that their overall range of values was slightly higher than those seen originally [b) c)].

3.3.1.3 PicoGreen assay

Figures 3.4, 3.5 and 3.6 all present results for double stranded DNA concentration obtained from PicoGreen assays, conducted on day 4 and day 7. At day 4 [figure 3.4 a)] MR01, 03, 05, 07 and 09 have a lower dsDNA concentration, indicating reduced cell number relative to the control. Other treatment values are like the control, although MR10 and 11 have a noticeably higher concentration. Of these differences, the decrease for MR09 is the only one which is statistically significant ($p<0.05$). At day 7 [b)] concentration values are generally increased, approximately clustering around 0.8 µg/ml instead of between 0.5 and 0.7 µg/ml as at day 4, demonstrating cell proliferation in all groups. MR05 shows the highest mean dsDNA concentration, though at this time point no statistically significant increases relative to the control were seen. Both MR01 and 09 had a significantly lower cell number ($p<0.05$) at day 7.

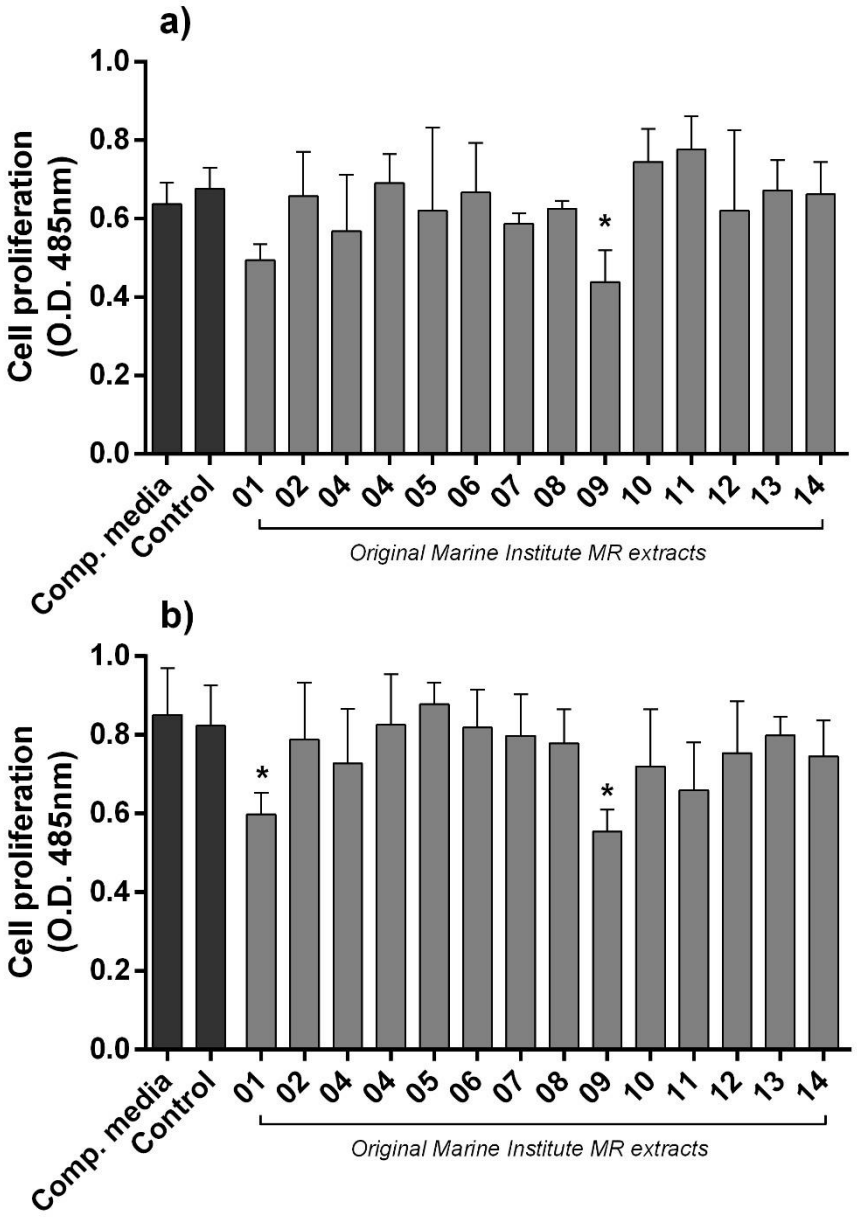


Figure 1.4: cell proliferation (PicoGreen assay) of hFOBs when challenged with extract set 1 (MR01-14). Cells were given a 24 h attachment period and then cultured with treatment for 4 [**a)**] or 7 [**b)**] days. Results are presented as the mean +/- standard deviation (n=4). *p<0.05 for treatment compared with to the control (complete media + 0.1% DMSO).

On day 4, except for MR-15, all treatments show lower dsDNA concentrations/cell number compared with the control group (figure 3.5). Of these, MR20, 21, 24 and 28 are significantly reduced ($p<0.05$). A similar distribution is seen at day 7 [b)], with MR15 the only treatment showing greater cell number than the control. The remaining extracts (excluding MR18) show lower cell number relative to the control group. For this time point MR17 and 27 are significantly reduced ($p<0.05$), though MR-20, 21, 24 and 28, as well as 23 and 26, also show pronounced decreases.

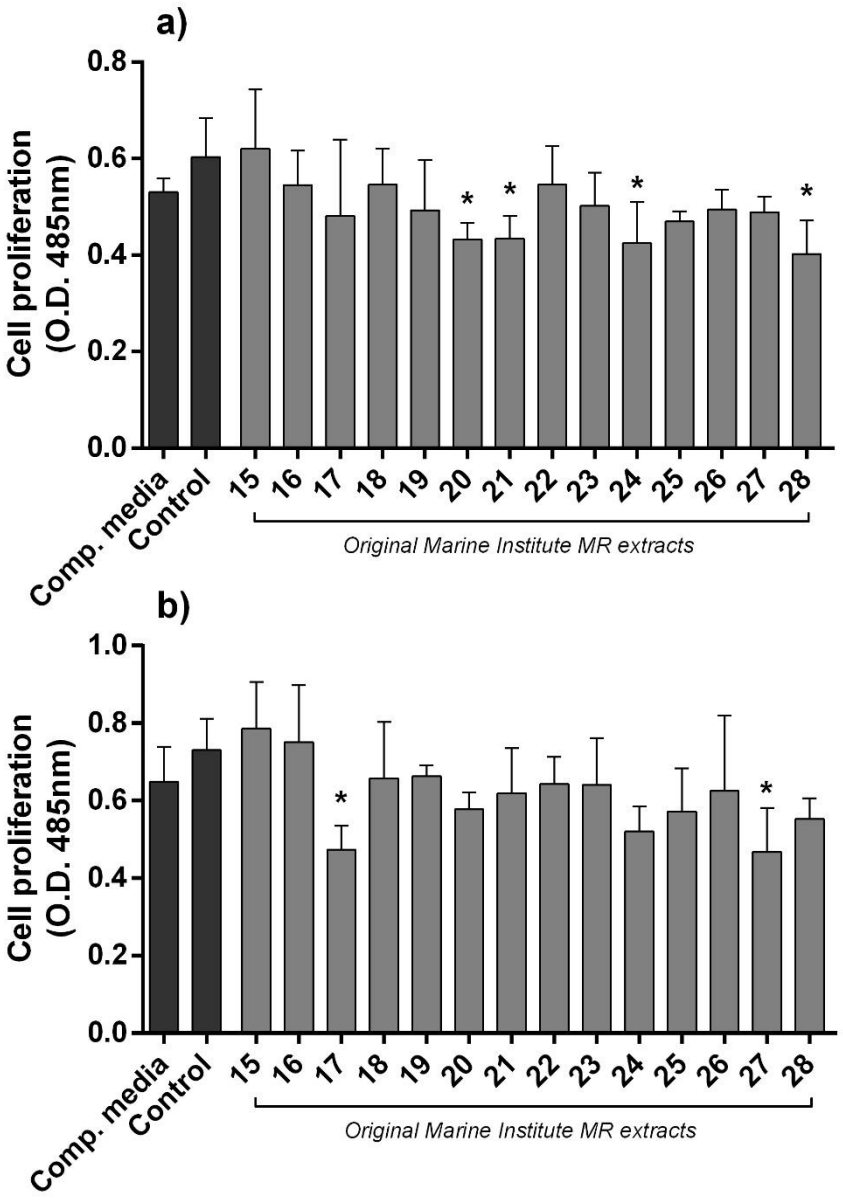


Figure 3.5: cell proliferation (PicoGreen assay) of hFOBs when challenged with extract set 2 (MR15-28). Cells were given a 24 h attachment period and then cultured with treatment for 4 [**a)**] or 7 [**b)**] days. Results are presented as the mean +/- standard deviation (n=4). *p<0.05 for treatment compared with the control (complete media + 0.1% DMSO).

Set 3 PicoGreen results (figure 3.6) were the most variable of the three, both in terms of standard deviation values and results between the two time points. Day 4 [a)] showed increases in dsDNA concentration/cell number for all treatments relative to the control group. These increases were smallest for extracts MR40 and 43. Statistically significant increases were seen for extracts MR29, 30, 32, 36, 37 and 38 ($p<0.05$).

Comparatively, all the control and test values were higher at day 7 [b)] than at day 4, ranging roughly between 0.5 and 0.7 µg/ml (compared to around 0.25 to 0.5 µg/ml). However, day 7 increases in treatment concentrations (indicating higher cell number) were less pronounced relative to the control, when compared to day 4. MR32 actually decreased in value, as did MR39 - although to a lesser extent. One significant increase was seen for MR33 (*P*=0.047).

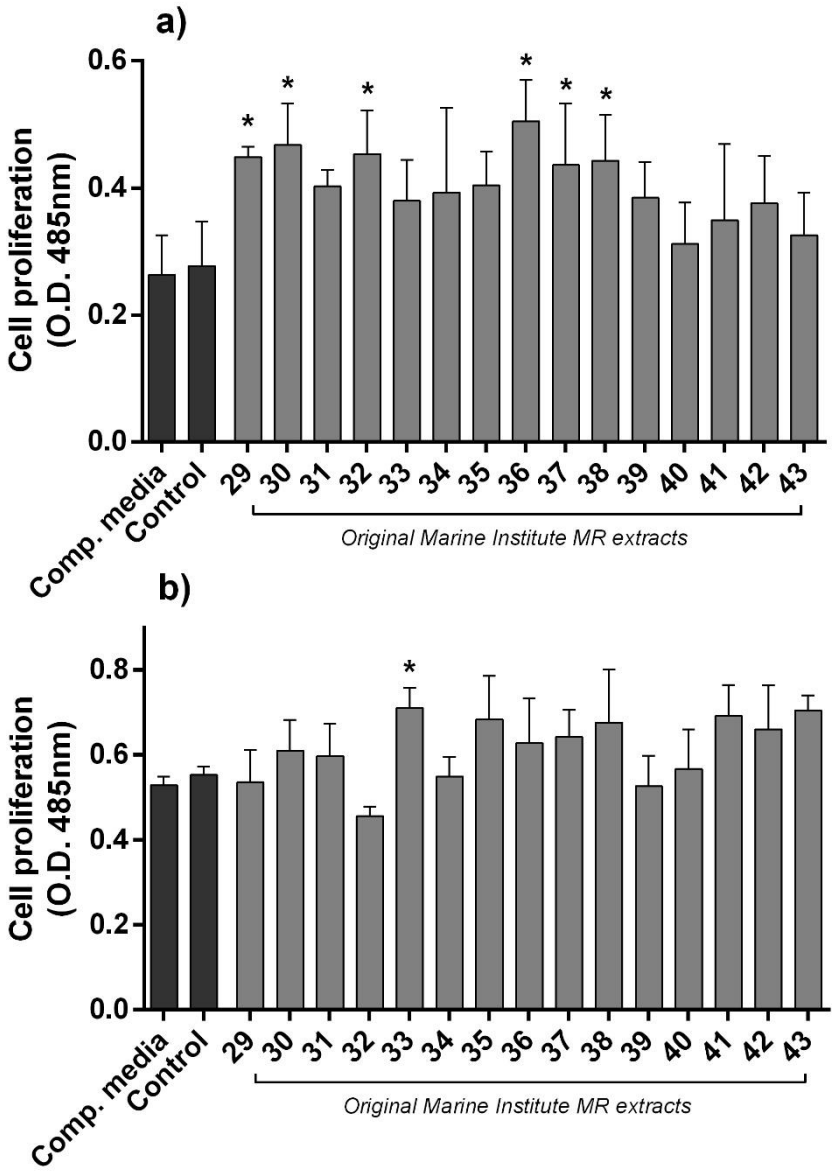


Figure 3.6: cell proliferation (PicoGreen assay) of hFOBs challenged with extract set 3 (MR29-43). Cells were given a 24 h attachment period and then cultured with treatment for 4 [**a)**] or 7 [**b)**] days. Results are presented as the mean +/- standard deviation (n=4). *p<0.05 for treatment compared with the control (complete media +0.1% DMSO).

3.3.1.4 Alkaline phosphatase assay

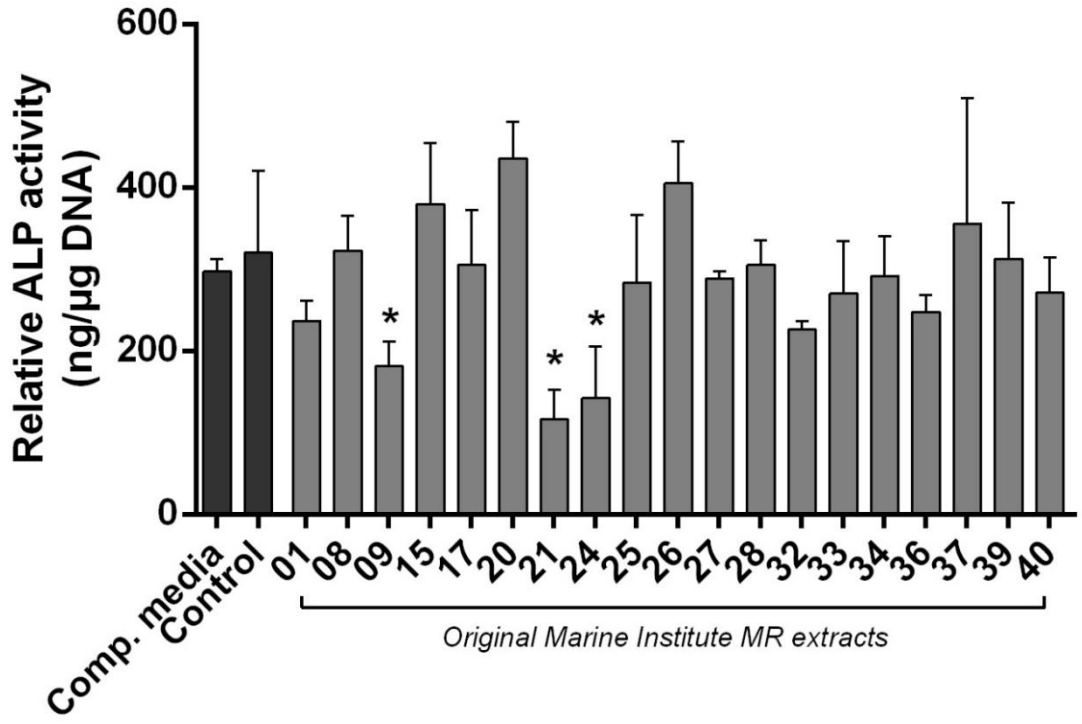


Figure 3.7: hBMSCs (donor:008, passage:4) differentiation at day 7 using ALP activity assay. Control treatment was osteogenic media with 0.1% DMSO, along with a complete media control for reference. Cells were challenged with a subset of extracts ranging between MR01-40. Cell differentiation is reported as ALP activity normalised to DNA concentration. Relative ALP activity is presented as mean +/- SD, (n=4). * indicates a significance difference ($p<0.05$) between the stated treatment and 0.1% DMSO control (dark grey).

ALP activity assay results generally show small deviations in activity values for DMSO dissolved extracts compared to control (figure 3.7). Of these, only extracts MR-09, 21 and 24 are significant, all causing substantial decreases in ALP activity level. MR-21 showed the greatest reduction, displaying a relative ALP activity value of only 116 ng/µg DNA, compared to a control value of 320 ng/µg DNA. Some of the remaining extract treatments produced noticeable reductions (e.g. MR-01, 32) or increases (e.g. MR-20, 27) compared to control, but these failed to reach statistical significance.

3.3.2 Batch of 25 extracts dissolved in ethanol

Ethanol was chosen for use as no reliable positive results for DMSO dissolved extracts were identified during previous screening. Specifically, ethanol was chosen over other potential solvents as it was shown to have a lower toxicity than DMSO during method optimisation work (chapter 2). LDH results are included for each of the 25 extracts that were dissolved in

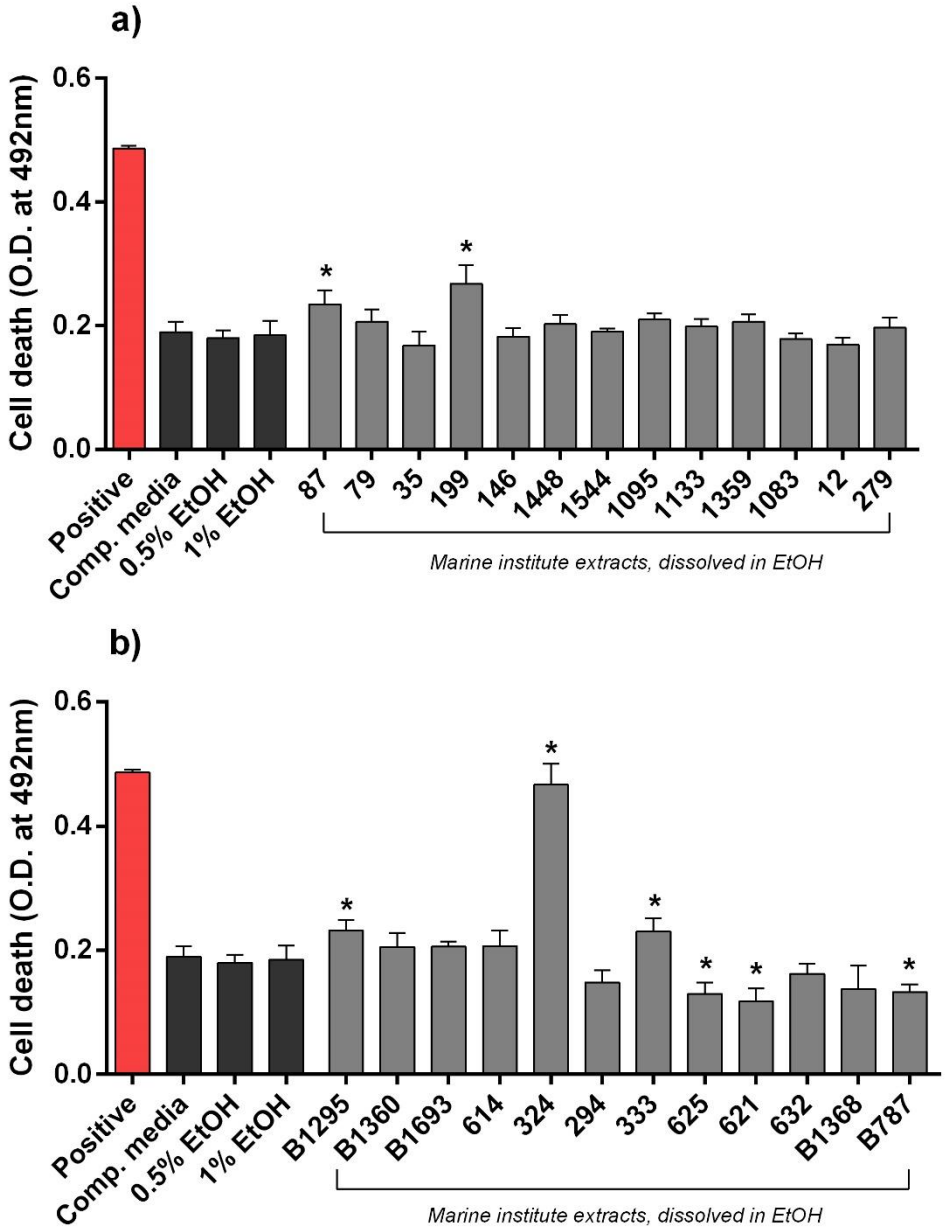


Figure 3.8: cytotoxicity results (LDH assay) for hFOBs challenged with 25 extracts (split between **a)** and **b)** for convenience) dissolved in ethanol and diluted in complete medium at a 0.5% concentration. Cells were given a 24 h attachment period, 24 h with treatment and then supernatant was collected for testing. Results are presented as the mean+/- standard deviation (n=4). C+ve (red) shows the absorbance values for 100% cell death. All values are corrected for background absorbance by subtracting appropriate negative control values, run on the same plate. Kit positive control confirmed the assay worked on each plate as expected. * $p<0.05$ for treatment compared to the 0.5% EtOH control (dark grey).

ethanol (figure 3.8). Kit reliability was again confirmed via inclusion of a positive kit control. Furthermore, the 100% cell death positive control was similar in value (0.4861) to those for the first 43 extracts tested (figure 3.3).

The complete media, 0.5% EtOH and 1% EtOH controls were similar in value, with approximately a 0.18 absorbance level. Most extracts treatments showed small fluctuations around these control values. However, extracts 87, 199, B1295 and 333 showed small but evident increases in cytotoxicity, which are all statistically significant ($p<0.05$). Also significant was extract 324, which had the greatest value of 0.467; almost equal to that of the 100% cell death positive control.

Decreases in absorbance value are also seen, including extracts 35, 1083, 12, 294, 625, 621, 632, B1368 and B787. For the first three extracts mentioned (35, 1083 and 12) these are small decreases, though the remainder are larger, particularly those of 625, 621, B1368 and B787. Of the decreases, those of extracts 625, 621 and B787 are statistically significant ($p<0.05$).

Cell proliferation values, as measured by CV assay, are presented as optical density readings, taken at the 585 nm wavelength (figures 3.9 and 3.10). Each plate (i.e. a) and b) - separate plates for each figure) had its own controls included to account for plate to plate variation in cell number. Control values are generally similar between plates, at just below a value of 0.9; except for the 0.5% EtOH control of figure 3.9 a), which is lower in value and has a greater standard deviation.

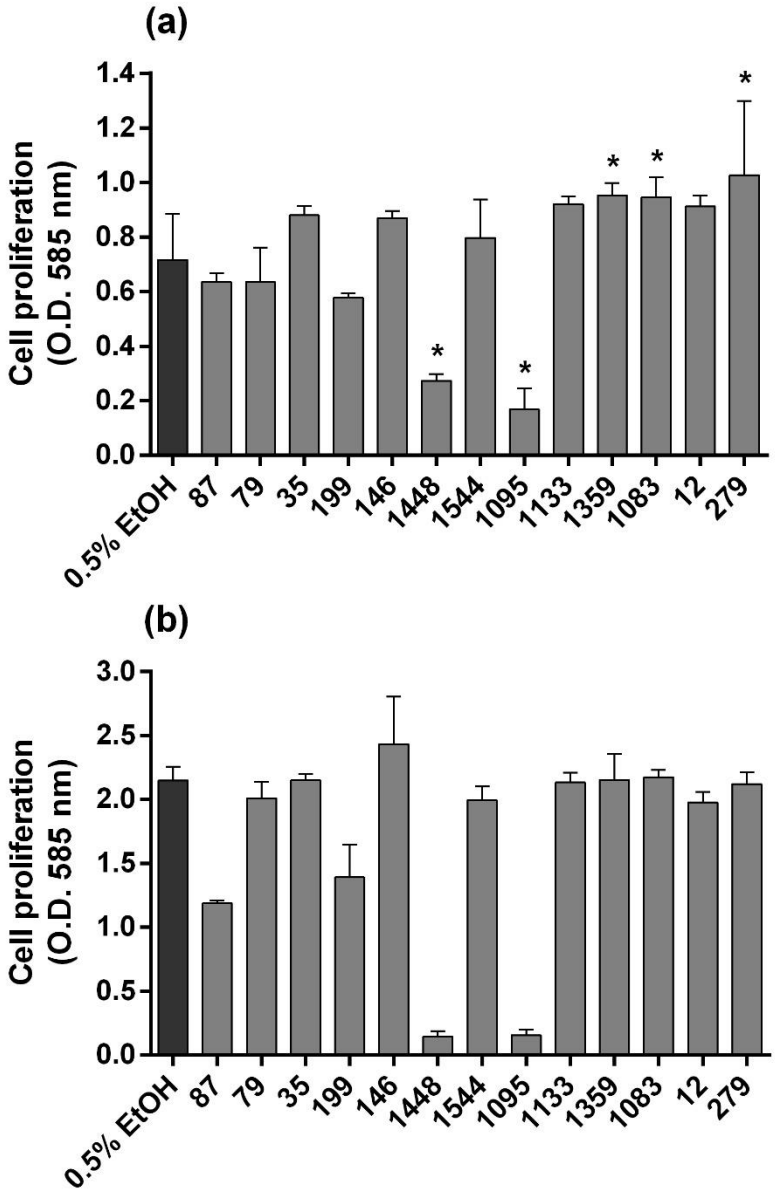


Figure 3.9: cell proliferation (crystal violet assay) of hFOBs challenged by 13 extracts (set 1) dissolved in ethanol and then diluted in complete media at a 0.5% concentration. Cells were given a 24 h attachment period and then cultured with the treatment for 4 [**a)**] or 7 [**b)**] days. Results are presented as the mean +/- standard deviation (n=4). *p<0.05 for treatment compared with the 0.5% EtOH control (dark grey).

At day 4 [figure 3.9 a)], small cell number decreases for extracts 87, 79 and 199 are seen in relation to the 0.5% EtOH control. Extracts 1448 and 1095 show an even greater and statistically significant reduction ($p<0.05$) in cell number. Remaining variation is less pronounced, though some small statistically significant increases are seen for extracts 1359, 1083 and 279. Day 7 [b)] results complement those of day 4, with most trends being maintained at the later time point. However, no deviations are statistically significant, as day 7 data did not have equal variance and was therefore analysed via Kruskal-Wallis test rather than One-Way ANOVA.

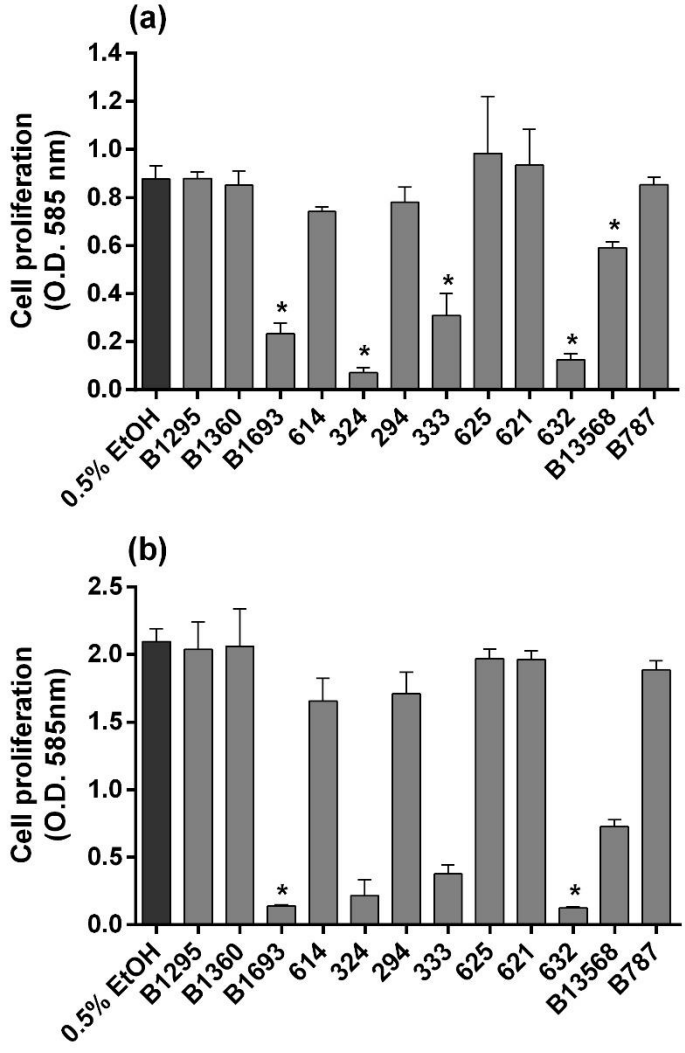


Figure 3.10: cell proliferation (crystal violet assay) of hFOBs challenged by 12 extracts (second set) dissolved in ethanol and then diluted in complete media at a 0.5% concentration. Cells were given a 24 h attachment period and then cultured with the treatment for 4 [**a)**] or 7 [**b)**] days. Results are presented as the mean +/- standard deviation (n=4). *$p<0.05$ for treatment compared with the 0.5% EtOH control (dark grey).

The second set of extracts (figure 3.10) are like the first, in that decreases in absorbance values are generally seen relative to the control, indicating reduced cell number. At day 4 extracts B1693, 324 and 632 show the largest reductions, 333 is slightly higher and B13568 is roughly two thirds the value of the control. All are statistically significant ($p<0.05$). Of the remaining extracts, 614, 294 and B787 show very small reductions in cell number whilst others are similar to the control. Day 4 trends are maintained at day 7, though there is a general increase in cell number with time. Previous significant reductions are still reduced, though are generally (with the exception of B1693 and 632) no longer significant despite being similar in value. This is again because day 7 data did not show equal variance and was therefore analysed using the Kruskal-Wallis test. The main change at this later time point is that extracts 614 and 294 are now significantly reduced relative to the control ($p<0.05$).

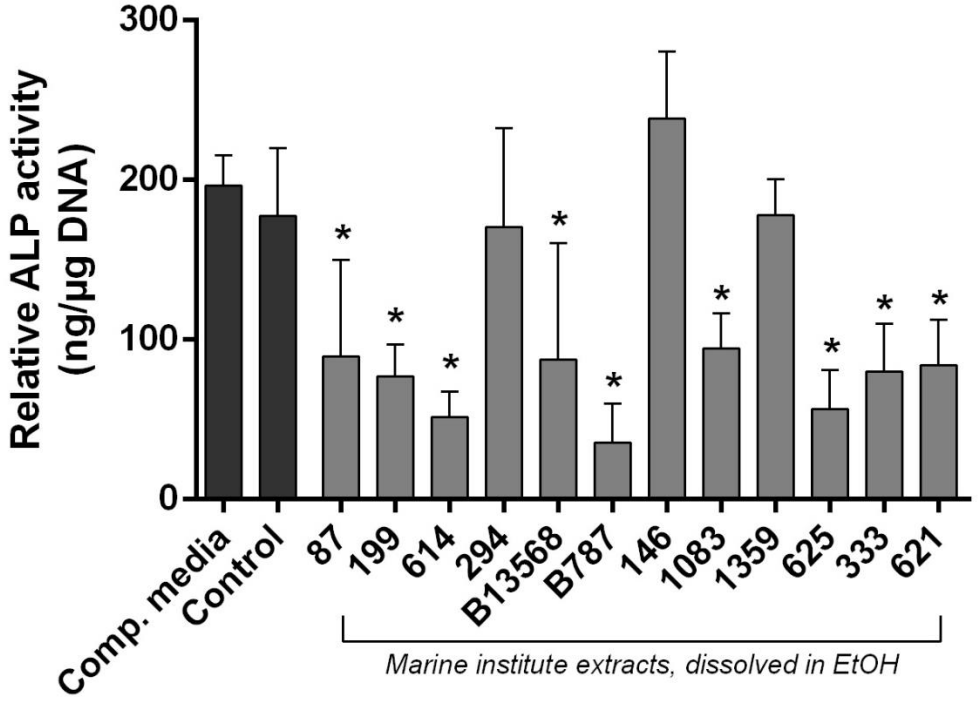


Figure 3.11: hBMSCs (donor:008, passage:4) differentiation at day 7 using alkaline phosphatase (ALP) activity assay. Control treatment was osteogenic media with 0.5% EtOH, along with a complete media control for reference. Cells were challenged with a subset of ethanol dissolved extracts at a 0.5% concentration. Cell differentiation is reported as ALP activity normalised to DNA concentration. Relative ALP activity is presented as mean +/- SD, (n=4). * indicates a significance difference ($p<0.05$) between the stated treatment and 0.5% EtOH control (dark grey).

ALP activity assay results of hBMSCs challenged with ethanol dissolved extracts (figure 3.11) show a similar trend to those of figure 3.7, with the only significant results being reductions (rather than increases) in ALP activity. However, these reductions are more numerous than those of figure 3.7, with only extracts 294, 146 and 1359 not significantly decreased relative

to control (0.5% EtOH). All decreases are similar in value between extracts, though extract B787 had the greatest effect – reducing ALP activity to value of approximately 35 ng/µg DNA, compared to 178 ng/µg DNA for control.

3.3.3 Powder extracts

Powder extract results are shown in figures 3.12-3.14. As there were so many different combinations of solvent type and concentration results have been separated for convenience. Figures 3.12 and 3.13 include control values (run on each plate) termed saline (0.1-10%), for comparison against extract values. Similarly, figure 3.14 includes saline controls relatable to HCl extractions, which were performed for extract 621. 621 was also extracted using 70% ethanol and 100% water, values for which - along with controls - are also shown in figure 3.14.

Firstly, 10% treatment with powder 625 reduced absorbance to a value of 0.21 at day 1 [figure 3.12 a)], relative to a control value of 0.31. This cell number reduction becomes more pronounced at days 4 and 7 [figure 3.11 a) and b) respectively]. Conversely, this powder at concentrations of 1% and 5% was increased relative to the control at all time points, At the lower concentrations of 0.5% and 0.1% there was less activity, though 0.5% was significantly increased at day 7. Comparatively, powder extract 294 showed less variation overall, with only the 5% treatment being increased from control values at each time point. 0.1% solution also showed an increase, but only at day 7. Finally, for 614 no concentration had any effect at day 1, but a bell-shaped response was seen at days 4 and 7. Of these 1% was particularly significant, having the greatest values at both time points - especially at day 7 where it was up 68% from the control.

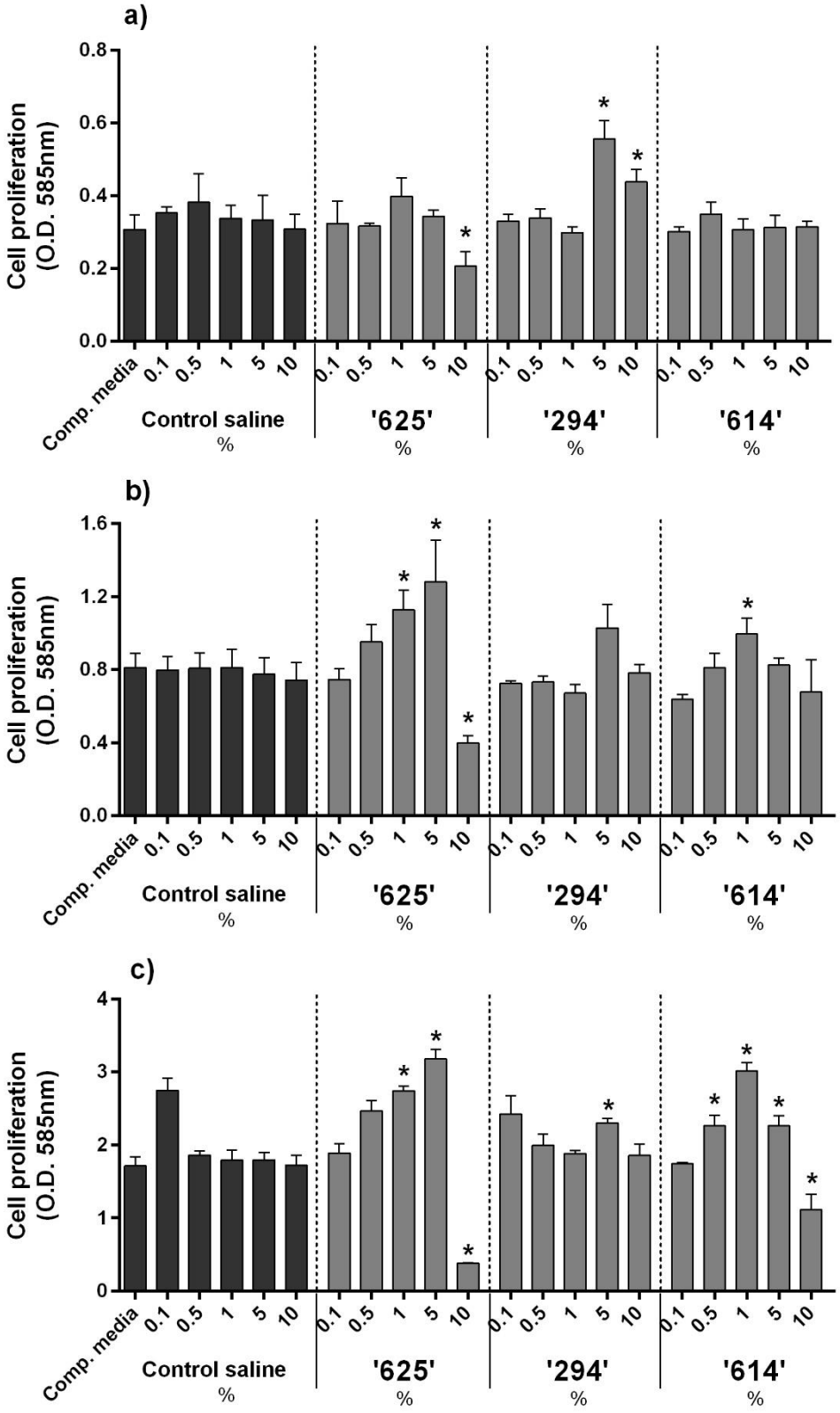


Figure 3.12: cell proliferation (crystal violet assay) of hFOBs challenged using powders 625, 294 and 614 dissolved in saline solution. Cells were given a 24 h attachment period and then cultured with the treatment for 1 [**a)**], 4 [**b)**] or 7 [**c)**] days. Results are presented as the mean +/- standard deviation (n=3). *P<0.05 for treatment compared with the correct saline control (dark grey).

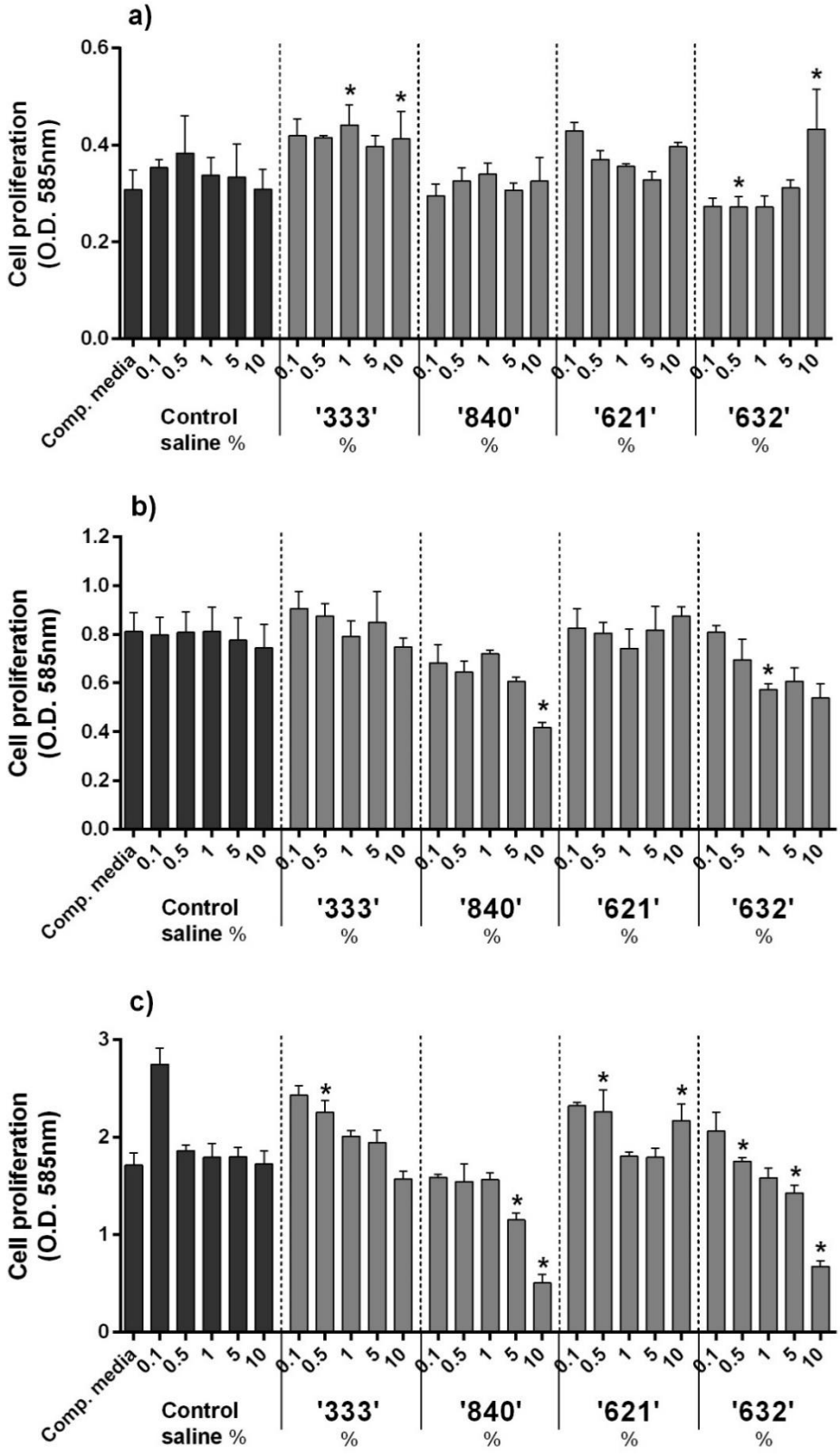


Figure 3.13: cell proliferation (crystal violet assay) of hFOBs challenged using powders 333, 840, 621 and 632 dissolved in saline solution. Cells were given a 24 h attachment period and then cultured with the treatment for 1 [**a)**], 4 [**b)**] or 7 [**c)**] days. Results are presented as the mean +/- standard deviation (n=3). *P<0.05 for treatment compared with the correct saline control (dark grey).

No clear trend can be seen at days 1 and 4 for powder 333 [figure 3.13 a) and b)], though by day 7 [figure 3.13 c)] there was a negative dose-dependent response. Here the lowest concentration powder solutions showed the greatest cell proliferation absorbance values, of which 0.5% was significantly increased from the controls. 840 showed a similar negative dose dependent response at days 4 and 7, though all treatments were reduced compared to controls, indicating low cell number. 621 on the other hand had a reversed bell shape, with the lowest absorbance values for mid-range concentrations of 1 and 5%. Conversely, 0.1, 0.5 and 10% showed small increases, of which 0.5 and 10% were significant ($p<0.05$), compared to the relative saline control at day 7. Finally, except for some variation at the day 1 time point, 632 showed a similar negative dose-dependent response to that described for 333 and 840. By day 7 only the 0.1% 632 powder treatment was similar in value to the control, whilst the others were all reduced.

For 621 'acid', where powder was first dissolved in HCl and then neutralised with NaOH, there's no clear relationship between time points [figure 3.14 a), b) and c)]. For days 1 and 4, 0.1 and 0.5% had the lowest absorbance values, though by day 7 a trend of decreasing absorbance with lower powder concentration is seen. For 621 dissolved in ethanol there was no significant deviation from controls (0.5% and 1% EtOH) at any time point. Finally, 621 water extraction values are shown, both for controls and treatments. Apart from day 4, water addition to medium decreased cell number in a dose dependent manner. At days 1 and 4, W621 showed no concentration response, though at day 7 high values were seen for 5 and 10% treatments. This was a significant rise from the relative controls ($p<0.05$), with the 10% treatment for example increased by 72%.

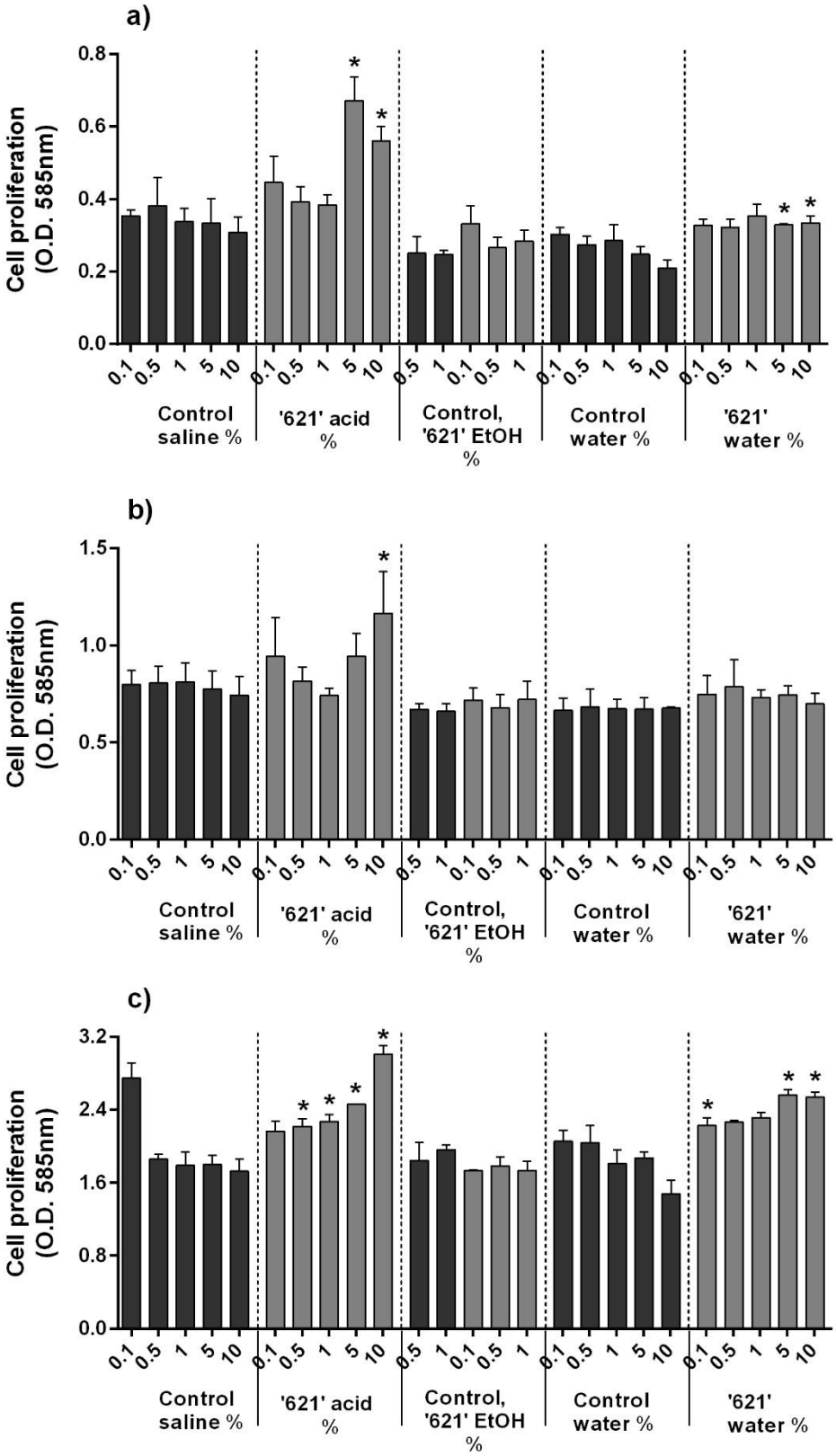


Figure 3.14: cell proliferation (crystal violet assay) of hFOBs challenged using powder 621 (along with relevant controls), dissolved using either an acid first saline extraction, ethanol or water. Cells were given a 24 h attachment period and then cultured with the treatment for 1 [**a)**], 4 [**b)**] or 7 [**c)**] days. Results are presented as the mean +/- standard deviation (n=3). *P<0.05 for treatment compared with the correct saline control (dark grey).

3.3.4 Summary tables of statistically significant results

Table 3.1: collation of results showing those treatments with statistically significant (p = <0.05) differences compared to the relevant control. This table contains information from set 1 (DMSO) and ethanol dissolved extracts. Those extract numbers highlighted in bold showed at least two different significant differences, either between time points or different types of assay. Green arrows represent a significant increase, whilst red indicates a significant decrease relative to the control.

Outcome measure	Extract	Increase / Decrease	Time pts (day)	Solvent	Conc. (%)
SET 1 EXTRACTS MR01-43					
XTT	MR08	↑	1	DMSO	0.1
(Cell	MR26	↓	1	DMSO	0.1
viability)	**MR32, 35, 36, 37, 39, 40**	↑	1	DMSO	0.1
LDH	MR04, 05, 13, 14	↓	1	DMSO	0.1
(Cell	MR15, 16, **17**, 18, 19, **20**, **21**	↓	1	DMSO	0.1
death)	22, 23, **24**, 25, 26, **27**, **28**	↓	1	DMSO	0.1
	MR **29, 30**, 31, **32**, **33**, 34	↓	1	DMSO	0.1
	35, 36, 37, 38, 39, 40, 41	↓	1	DMSO	0.1
	42, 43	↓	1	DMSO	0.1
PicoGreen	MR09	↓	4	DMSO	0.1
(Cell	MR01, 09	↓	7	DMSO	0.1
proliferation)	**MR20, 21, 24, 28**	↓	4	DMSO	0.1
	MR17, 27	↓	7	DMSO	0.1
	MR 29, 30, 32, 36, 37, 38	↑	4	DMSO	0.1
	MR33	↑	7	DMSO	0.1
BATCH OF 25 EtOH EXTRACTS					
LDH	**87, 199**, B1295, **324, 333**	↑	1	EtOH	0.5
(Cell	625, 621, B787	↓	1	EtOH	0.5
death)					
Crystal V.	**1448, 1095, B1693, 324, 333**	↓	4	EtOH	0.5
(Cell	**632, B13568**	↓	4		
proliferation)	1359, 1083, 279	↑	4	EtOH	0.5
	87, 199, 1448, 1095	↓	7	EtOH	0.5
	B1693, 614, **324**, 294, **333**	↓	7	EtOH	0.5
	632, B13568	↓	7	EtOH	0.5

Table 3.2: collation of results showing those treatments with statistically significant (p = <0.05) differences compared to the relevant control. This table contains information from powder extracts. Those extract numbers highlighted in bold showed at least two different significant differences, either between time points or different types of assay. Green arrows represent a significant increase, whilst red indicates a significant decrease relative to the control.

Outcome measure	Extract	Increase / Decrease	Time pts (day)	Solvent	Conc. (%)
POWDER EXTRACTS					
Crystal V.	625	↓	1, 4 ,7	Alkaline	10
(Cell	625	↑	4, 7	Alkaline	5 + 1
proliferation)	294	↑	1	Alkaline	10
	294	↑	1,7	Alkaline	5
	614	↑	4,7	Alkaline	1
	614	↑	7	Alkaline	5 + 1 + 0.5
	614	↓	7	Alkaline	10
	333	↑	1	Alkaline	10 + 1
	333	↑	7	Alkaline	0.5
	840	↓	4	Alkaline	10
	840	↓	7	Alkaline	10 + 5
	621	↑	7	Alkaline	10 + 0.5
	632	↑	1	Alkaline	10
	632	↓	1, 7	Alkaline	0.5
	632	↓	4	Alkaline	1
	632	↓	7	Alkaline	10 + 5
	H621	↑	1, 4, 7	Alkaline	10
	H621	↑	1, 7	Alkaline	5
	H621	↑	7	Alkaline	1 + 0.5
	W621	↑	1, 7	Alkaline	10 + 5
	W621	↑	7	Alkaline	0.1

3.4 Discussion

Experimental work detailed within this chapter aimed to assess the impact of crude extracts on both cell death and cell proliferation (as well as brief ALP activity [cell differentiation] testing). This was achieved for DCM/methanol extracts reconstituted in DMSO or ethanol, as well as for powder residue extracts.

3.4.1 DMSO and ethanol extracts

3.4.1.1 Decreased cell proliferation

XTT results, whilst not specifically measuring cell proliferation, do give a good indication of this variable, as they represent the number of metabolically active cells able to produce the formazan product. MR-26 was the only extract which showed a significant reduction in XTT value, as ethanol dissolved extracts or powder residues were not tested with this assay. Additionally, MR-26 also gave relatively low dsDNA concentration from PicoGreen results. PicoGreen gives a quantitative indication of cell number, as with more cells/greater proliferation a higher dsDNA concentration would be expected. These results may therefore indicate the presence of a specific bioactive, or bioactives, which reduces proliferation. Of note, this was the only instance in which an XTT value correlated with dsDNA concentration, suggesting that extract-based effects on metabolic activity at this early time point were generally not maintained when measuring proliferation at days 4 and 7.

Many other extracts, both those dissolved in DMSO and ethanol, showed reduced cell proliferation values during seven days in culture including: MR01, 09, 20, 21, 24, 26 and 28, as well as ethanol-based extracts 1448, 1095, B1693, 324, 333, 632, B13568, 87, 199, 1448, 1095, B1693, 614 and 294.

Osteoblasts undergo a sequential development which can be ordered into three main stages: a proliferative period, ECM development and finally mineralization of this matrix. Freshly seeded osteoblasts tend to be in the proliferative stage, actively undergoing mitosis to increase cell number, supported by ample culture space and high nutrient levels. However, as osteoblasts develop they begin to differentiate towards the mature phenotype and proliferation is reduced (Aubin 2001; Lian & Stein 1995). It may be that the extract mixes detailed above suppressed proliferation relative to other treatments and controls. Alternatively, they may have supported earlier cell differentiation at the cost of reduced cell division. To test this hypothesis a small ALP assay was conducted, focusing on those extracts which reduced cell proliferation. As previously described, no extract showed a significant

promotion of ALP activity and instead significant reductions were common. This indicates that those extracts which suppressed proliferation were not also supporting cell maturation. To note, both ALP assays made use of hBMSCs rather than hFOBs, as opposed to other work detailed within this chapter. This was a purposeful decision, as hBMSCs previously displayed better ALP activity than hFOBs during optimisation testing (chapter 2, section 2.7.1). As only a limited number of promising extracts were being tested, and as this was the last measure of potential cell activity planned for both extract sets (due to their limited proliferation response), hBMSCs gave the best chance of detecting bioactivity.

Another important factor to consider when discussing proliferation is cytotoxicity, as increased cell death levels would naturally also result in a lower cell number. Before comparing results, it should be noted that obvious differences in LDH values existed between DMSO and ethanol-based experiments, as seen in figures 3.3 and 3.7. Figure 3.3 readings, for extracts MR 01-43, were approximately 10 times lower than those for ethanol dissolved extracts. However, this was also the case for medium only control treatments, indicating higher reading values were not due to extract inclusion, or use of ethanol as a solvent. Similarly, this change was unlikely to be due to human error, as cell seeding density was kept the same; as confirmed by comparable positive control values between the two experiments. Instead these differences were more likely a feature of the FBS used during culturing for each test. Originally FBS from PAA was used until it ran out, after which a lot from Sigma-Aldrich proved most effective in batch testing. Any use of FBS is known to have a stimulatory effect on LDH activity (Baba et al. 2005), and as the new batch was first used just before these results were obtained it is likely the cause of variation.

All treatments dissolved in DMSO which lowered cell proliferation also had low or significantly reduced cytotoxicity levels, in comparison with controls. This supports the idea that these mixes contain bioactives able to suppress cell proliferation or promote cell maturation, rather than toxic compounds. However, there were instances where toxicity increased whilst proliferation values decreased, particularly those obtained using the crystal violet assay. 324 for example - which was completely toxic - showed very little proliferative activity, indicating no cells were present to be stained by the dye. Extract 87 and 199's lower proliferation values (compared to controls) may also be explained through a slight toxic effect, as ALP results confirm it isn't linked to an increase in differentiation. However, there are also results which do not follow the 'expected' trends based on both LDH readings and observations. 632 is a good example of this, as it presented an LDH value lower than that of controls, but also a very low cell proliferation value of 0.12 at both day 4 and 7. In fact, at

both time points 632 had an optical density almost identical to that of 324, which as mentioned was highly toxic. In this instance, it seems evident that 632 was having a toxic impact on cells, but perhaps this effect was only seen at the later time points of day 4 and 7; for example due to stimulation of apoptotic pathways which had a delayed effect. Extracts 1448 and 1095 also showed the same trend of a very low O.D. value at day 7, as did B1693; which may again indicate a late toxic effect.

Some extracts had no impact on proliferation but presented reduced cytotoxicity levels, such as 625, 621 and B787. This may again suggest the presence of a compound, or compounds, able to reduce cell death. Such an effect could be through repression of apoptotic pathways, or could have been related to cell differentiation. However, significant reductions in ALP activity were seen with all three of these treatments, ruling out this hypothesis. Overall, decreases in proliferation are common throughout this work. However, at this stage in testing, extracts were being sought which promoted cell proliferation or differentiation, or both. In a treatment situation this could potentially yield a larger number of osteoblasts, or facilitate precursor development to the mature phenotype, improving bone formation and healing rates.

3.4.1.2 Increased cell proliferation

Treatments which increased hFOB proliferation were less common that those which decreased it, though there are still numerous examples to discuss. Extracts 10 and 11 for example, increased the proliferative capacity of cells, explaining their noticeably higher dsDNA concentration values at day 4. Increased dsDNA concentration was likewise present at days 4 and 7 for MR15, which also shows a very low LDH absorbance value. This might therefore indicate the presence of a bioactive able to stimulate cell proliferation whilst reducing cell death, or at least having limited cytotoxicity. Similarly, extracts MR29-43, 625, 621 and B787 all coupled their significant reductions in cell death with average to high proliferation values, which was as expected if cells are healthy.

Small LDH test values are not uncommon, especially when low concentrations of natural compounds are applied to cells. For example, no LDH release could be observed in osteoblasts treated with lactic and glycolic acid until concentrations of approximately 100 mM were reached (Meyer et al. 2012). Taking MR-29-43 as an example, even the highest LDH absorbance levels in this group, those of MR31 and 32, do not appear to have impacted early cell numbers or proliferation. For instance, MR32 showed a significant increase in cell viability

(XTT) and MR31 was still higher in value than the control. Also significant in terms of increasing cell viability were MR35, 36, 37, 39 and 40, all of which also showed average to low absorbance values in the LDH assay. These day 1 tests therefore support the presence of non-toxic compounds able to increase cell metabolic activity in those extracts mentioned. Furthermore, PicoGreen results indicate this stimulative effect was maintained at later time points, particularly at day 4 where most treatment values were noticeably higher compared to the control.

3.4.1.3 Effects between time points

Treatment effects determined by an assay are often maintained between time points, which is as expected if an active compound retains its bioactivity over time. Extracts 32, 36, 37 and 38 for example showed sustained increases in cell activity over days 1, 4 and 7. Furthermore, extracts 625, 621 and B787 all coupled their significant reductions in cell death with sustained, but fairly average, proliferation values. Significant inhibitions of cell proliferation were also sustained over time in this work, such as for extracts 20, 21, 24, 28, 1448, 1095, B1693, 324, 333, 632 and B13568.

There are however instances where effects were not maintained and instead changed between time points. For example, MR10 and 11 appear to have increased the proliferative capacity of cells, explaining their noticeably higher dsDNA concentration values at day 4. However, this increase in concentration was not mirrored with the same extract treatments at day 7. This could mean that a potential stimulatory effect of the extract was limited to early time points, or it may have been a feature of contact inhibition. Contact inhibition, where a cell stops division when touching another cell or object, is a feature of normal, healthy eukaryotic cells (Abercrombie 1979). Furthermore, it is a likely limit to cell growth and development within the small well area of assay plates, which would explain why DNA concentration values rarely exceeded 0.8 µg/ml for any time point/treatment.

Alternatively, increases in proliferation can lose their significance at day 7, such as with extracts 1359, 1083 and 279. However, for these treatments it is likely that original significant results may have been influenced by the relatively low value for the ethanol and medium control at day 4, rather than extract activity. Finally, inhibition of proliferation also showed changes between time points, such as for MR17, 27, 87, 199, 614 and 294. These extracts had average-low cell proliferation values at day 4, which only became significantly reduced at day 7, indicating a late reduction effect of active compounds.

One particularly interesting example of time point variation is that of MR32, which showed the lowest dsDNA concentration value (0.46 µg/ml) at day 7, a trait at odds with the significant increase at day 4. As previously discussed this may indicate the extract effect was limited to earlier time points, though it may also be a function of the crude nature of extracts used in these experiments. For example, processing steps were limited during extract preparation, both in production of the dried material and its reconstitution in a solvent. As a result, each extract likely contained a mix of different proteins, nutrients and other molecules, which may have had a range of effects on cell cultures. Competing extract effects could therefore explain the results of MR32, with a bioactive stimulating proliferation at day 4 outcompeted by one facilitating cell differentiation at day 7. Without knowing more about the composition of each extract it is difficult to confirm this, though competition between different bioactives is commonly reported in other work. For example, out of four proteins isolated from the water soluble matrix (nacre) of *Pinctada maxima,* three were shown to increase ALP activity - whilst one decreased it (Almeida et al. 2000). In this instance, a net positive effect on mineralisation was observed, coupled with a decrease in cell number characteristic of greater cell differentiation (Almeida et al. 2000). Presumably, if a greater proportion of proteins were present which reduced ALP activity then mineralisation would not have been enhanced. However, based on the difficulty of separating and characterising active molecules many extracts tested within this field are crude, with studies reporting overall effects rather than those specific to one molecule.

3.4.2 Issues surrounding solvent choice

Processing/production of DMSO and ethanol dissolved extracts was very similar. The main difference (chapter 2) is that ethanol is tolerated by cells at higher concentrations than DMSO. Though some small variation in efficiency is likely, ethanol appears to dissolve extracts as effectively as DMSO. It was therefore chosen as a replacement solvent as it allows a higher treatment concentration to be achieved, generally increasing the likelihood of seeing an extract effect.

Whilst some interesting trends were found using DMSO and ethanol extractions, no large-scale increases in proliferation were observed. Therefore, it was decided that powder residues - left over from the original DCM/methanol extraction - would also be investigated for potential activity. Previous oily residues from combined DCM and methanol extractions were relatively difficult to dissolve, even in strong solvents such as DMSO. However, powders

have the benefit of a much greater surface area, and thus a weaker solvent starting point was chosen: 0.1M NaOH. This basic solution has a high polarity - similar to water - and would therefore be expected to dissolve other polar or ionic molecules. Its most important benefit is that, once properly neutralised, treatments are dissolved in saline solution and should have a very limited toxic effect; allowing high concentrations to be used (up to 10%), even greater than those used with ethanol.

Dissolution was achieved, using the method described in 3.2.2.3, and was evidenced by colour change in test solutions over time. However, varying degrees of powdered material did remain undissolved and therefore solutions were centrifuged before use, to limit addition of this to the test plates. Unfortunately, centrifugation alone was not sufficient to remove all this residue and, as mentioned in section 2.4.2, leftover material can interfere with results of both LDH and crystal violet assays. As such, powder residue results cannot be trusted completely, though in many instances residue was minimal and experimental work still gave a useful indication of extract bioactivity.

3.4.3 Powder extracts

Grouping the extracts together, there seems to be three sets: those that caused no increase in cell activity; those that had a linear dose response in either direction; and finally those which had a bell-shaped response curve.

Powder residues 840, 632 and E621 (dissolved in 70% ethanol) made up the first group. 840 and 632 reduced proliferation in a dose dependent manner. LDH results for this experiment were limited due to the residue issue, meaning only supernatant from a reduced number of low concentration treatments was included for testing. As such, toxicity was hard to confirm and these results have not been included, though a slight increase in cell death level was observed at 0.1% for treatment 840. It may therefore be the case that proliferation was limited through cell death, or a bioactive induced repression of cell division.

Lack of activity for ethanol 621 extract is particularly interesting, as there did appear to be a stimulatory effect of high concentrations of 621 dissolved in other solvents. Water 621 extract was a good example of this, as cell activity at 10% (day 7) was increased from controls, whilst observations confirmed residue was limited in this treatment. As water is a more polar solvent than ethanol this suggests the active component(s) of 621 is also polar, such as a protein with exposed groups and a high dipole moment. Acid (0.1M HCL neutralised with NaOH) 621 extract also showed the typical pattern of dose response for a treatment which

is known to have a positive effect, with greater cell response at higher doses. However, observations showed that residue was significant for the acid extraction and therefore this will not be considered as an indication of bioactivity. Powder extract 333 was also part of the second group, showing activity at lower concentrations of 0.1 and 0.5%, indicating it contains a compound able to stimulate cell activity at a low level. It also likely contains another compound, or compounds, which repress proliferation at higher concentrations. The same may also be true for 294, which, apart from an increase at 5% (likely caused by residue), showed similar small-scale increases at 0.1/0.5%.

625 and 614's bell shaped proliferation response to treatment is particularly interesting. For powder 625 there was obvious residue present which could have led to false positive proliferation values. Nonetheless, observations on all days indicated that cells were present and healthy, even at the maximum 5% concentration, making this a good candidate for further investigation. Finally, 614 was the most promising and interesting powder tested. Its peak activity at 1% indicates it may contain multiple bioactives. Lower than this, not enough compound may have been present to stimulate cells, whilst above this level another repressive molecule could have been obscuring its positive influence.

3.5 Conclusion

A succinct summary of testing on so many extracts is difficult, as each presents different trends, promises and challenges. The first set of tests, conducted on extracts 1-43, was limited by the toxicity of DMSO and very low levels of material available for testing. Some interesting trends were apparent, including wide spread low toxicity and sustained changes in cell proliferation; however, these changes were often small, repeatability was a challenge due to issues with PicoGreen reliability and, as stated, amounts of test material were low. The latter is most concerning, as it is unlikely that further detailed tests could be conducted. Raw extracts dissolved in EtOH were more repeatable, showing sustained decreases in cell proliferation, both over time and between assays. However, these too were limited by extract availability. On the other hand, much more material was available for powder residues left over from the original extraction process, making these more suitable for long term testing. Despite being the 'most' processed, in terms of components removed by DCM and methanol, these extracts resulted in relatively large and significant changes to cell proliferation. As such, powder extracts showed the most promising activity of extracts detailed within this chapter, indicating good osteogenic potential. Future work needs to test powder extracts prepared using the refined extraction method, which eliminates residue as

a confounding variable. Furthermore, activity should be confirmed using primary hBMSCs - which are more representative of cells within the human body and therefore give a better indication of an extracts pre-clinical potential.

Chapter 4

Powder extract potential

4.1 Introduction

Milling, lyophilization or mechanical degradation of extracts to create powdered material is common in natural product testing. These processes serve to break up large material and increase the overall surface area of an extract, aiding solvent dissolution. One simple example is a method used to create a seed powder extract (Jagtap & Bapat 2013), where seeds were simply dried, ground and extracted in water. Likewise, extracts supplied for use in this study (by the Marine Institute, Galway) were all treated with a similar initial method (see chapter 3), being freeze dried and milled to generate a fine powder - before organic solvent extraction (Rae et al. 2013). Most studies take this approach and focus on testing fractions which dissolve in an organic solvent(s), as (depending on solvent choice) these fractions tend to contain the most organic material and therefore give the best chance of detecting bioactivity. This was the rationale behind sequential testing used in this study, which focused first on material which dissolved in original DCM/methanol extractions. However, residue left over from these extractions still presents a viable source of natural products/compounds and therefore should also be tested for bioactivity. Therefore, this chapter - and much of the work within this thesis - focuses on describing the bioactive potential of 'powder extracts'. These are (as described in chapter 3) the material retained after solvent extraction, which to date have seen relatively little focus in natural product research – often being discarded rather than tested (Jagtap & Bapat 2013).

Many published studies which use the terms 'powders' or 'residues' refer to efforts to produce value-added products. For example, in one study residues left over after the juicing of star fruit were then extracted with acetone, ethanol and water. These extractions were shown to have greater antioxidant potential than the juice fraction which is traditionally consumed (Guanghou & Lai 2006). Similar work tested milled powder which was left over during the processing of Mate (a species of the holly family), which is commonly consumed as a beverage in South America. Again, residue material showed excellent properties – having a high polyphenol content and good antioxidant potential (Vieira et al. 2010). In addition to these value-added approaches there are also studies which include residue testing during their evaluation of natural products, with varying degrees of success. For example, extraction residues made during the production of lipids from the mussel *Perna canaliculus* showed no activity as a potential osteoarthritis treatment (Gibson & Gibson 1998). Similarly, aqueous residues with low activity levels were produced after extractions using *n*-hexane, ethyl acetate and 1-butanol - on the red alga *Polysiphonia urceolata* (Duan et al. 2006) and the seaweed *Fucus vesiculosus* (T. Wang et al. 2012). In both studies the residues showed

moderate antioxidant capacity which generally did not exceed that seen with the organic solvent fractions. However, there are examples where residue material extractions show good bioactive properties. For example, ether extraction of a residue produced from an alcohol extract of *Mormodica charantia* (commonly known as bitter lemon) shows hypoglycaemic activity (Srivastava et al. 1993), as do extracts from seeds of the plant Fenugreek. Here all components had a positive effect on diabetic model rates, including the original powder from milling, a methanol extraction and residue from this extraction.

Despite the variability of residue activity reported in different studies, they were still deemed to be important for investigation in this work for multiple reasons. Firstly, aforementioned studies showed that residue extractions often contain significant organic material, which may have activity potential. Interestingly, reports of osteogenic potential for these extracts was also very limited, increasing the novelty of this work. Furthermore, in the present study there was a large reserve of powder extracts available for testing, meaning most experiments wouldn't be limited by available material. Finally, upon initial screening (see chapter 3) powder extracts showed the best activity potential – highlighting the need for more detailed testing. This chapter will determine powder extract activity using hBMSCs, specifically focusing on their cell proliferation, differentiation and mineralisation potential. As described in chapter 2, primary cells like hBMSCs are more representative of the cells within the human body and therefore give a better indication of future treatment potential. These cells also displayed more repeatable patterns of cell growth and better differentiation potential when compared to hFOBs (chapter 2), as shown by their high ALP expression and superior mineralisation levels.

4.2 Methods

4.2.1 Samples used in powder extract preparation

Throughout this chapter extracts from a total of 14 different species were tested, the identification information for which is shown in table 4.1.

Table 4.1: identification information for all the samples used to produce powder extracts. Included are the original identification (ID) numbers, genus and species, taxa (a general description of the sample material), sample/ID dates and sample location. Where species is not known 'sp.' is written. For sample 614, * denotes that another two samples from the same species were also collected for 'in-house' extraction. These included intertidal material from Donegal and Christchurch Harbour, Dorset.

ID	Genus, species or description	Taxa	Sample date	Location
614*	*Ceramium secundatum*	Red alga	05-Jun-12	Finavarra; Co. Clare
333	*Plocamium lyngbyanum*	Red alga	27-Aug-11	Carraroe; Co. Galway
334	*Plocamium cartilagineum*	Red alga	05-Sep-16	Donegal and Dorset
625	*Osmundea sp.*	Red alga	21-May-12	Finavarra; Co. Clare
294	*Boergeseniella fruticulosa*	Red alga	07-Mar-11	Spiddal ; Co. Galway
615	*Ceramium pallidum*	Red alga	05-Jun-12	Finavarra; Co. Clare
621	*Punctaria sp.* (order: Ectocarpales)	Brown alga	21-May-12	Finavarra; Co. Clare
840	*Sargassum muticum*	Brown alga	02-Mar-14	Doaghbeg, Fanad, Donegal
632	*Cladostephus spongiosus*	Brown alga	20-Aug-12	Finavarra; Co. Clare
1358	*Psolus squamatus*	Deep sea sponge	07-Jun-13	Cruise 13008
137	Genus: *Lophelia*	Coral	N/A	N/A
792	*Porphyra umbilicalis*	Red algae phylum of laver species	N/A	N/A
793	*Porphyra linearis*	As above	N/A	N/A
698	*Percursaria percursa*	Seaweed, family Ulvaceae	N/A	N/A

4.2.2 Powder extract preparation

4.2.2.1 Powder production at the MI

Powder residues were originally produced during extraction at the Marine Institute in Galway (for details see chapter 3, section 3.2.1). However, fresh algal material was also collected and extracted solely for use in this study, using an adapted version of the original extraction method.

4.2.2.2 Powder production method for 'in-house' extractions

In order to test the reproducibility of results, fresh samples were collected for extraction from two species of epiphytic red algae: *Ceramium secundatum* and *Plocamium cartilagineum*. Samples from both species were collected during September 2016 from two intertidal locations – Christchurch Harbour, Dorset (UK) and Donegal, Ulster (Ireland). After collection, samples were kept in seawater during transport and immediately frozen upon arrival at the laboratory (–20°C). As stated, the method used followed that of the Marine Institute, to limit variation as much as possible between extractions. Briefly, 2 – 3.60 g of each algae (from either UK or Irish sample locations) was cleaned of epiphytes by hand using fresh water and placed into a Savant Modulyo freeze dryer (USA) with an Edwards RV8 vacuum pump (USA).

Drying took approximately 3 days, after which each sample was extracted with DCM at room temperature for 24 h (with stirring) – at an approximate concentration of 100 ml solvent/1 g sample. Dissolved material was then removed via filtration (Whatman 150 mm filter paper), placed in a flask and left to air dry, whilst un-dissolved residue was collected separately. Residue was then treated with methanol for 24 h (room temperature, stirring) and remaining undissolved material was again collected. Solvent extraction left behind 65 – 73% of the original starting sample weight as a powder residue. Powders were left on a tray to evaporate off any residual solvent, before being weighed, foil packed and frozen (–20°C). This entire extraction method was also repeated, using ethanol as a solvent – yielding an ethanol powder residue and dissolved extract for each sample. This new ethanol extraction was included to test if use of only one solvent, as opposed to DCM and MeOH, would impact extract activity.

4.2.2.3 Powder dissolution to form extracts

As represented in figures 4.1 and 4.2, powder residues were added to either 0.1 M NaOH or water at a concentration of 30 mg/ml. This mixture was then subjected to 3 cycles of vortexing, placement on a rotary shaker (45 min), heating to 37°C (45 min) and sonication (25 min) – using a low frequency sonicator (Branson 3510, USA) with the addition of ice to stop heating > 37°C. After dissolution, suspensions were then centrifuged (Heraeus Primo R, ThermoFisher Scientific, UK) for 15 min at 3000 g. Subsequently, supernatant was transferred to a fresh falcon tube and centrifuged again, for a further 15 min (3000 g) – before being collected once more and transferred to a new falcon tube. The resultant solution was

neutralised (approximately pH 7.5) via the addition of 0.5 M HCl, using a pH meter (meter: Jenway 3051, UK; probe: Orion™ PerpHecT™ ROSS™ Combination pH Micro Electrode, ThermoFisher Scientific, UK). This was most crucial for solutions containing 0.1 M NaOH, as water based extractions rarely varied in pH level. For alkaline extractions this neutralisation created a 100 mM NaCl solution, containing dissolved powder extract material. For each extraction changes in colouration, smell and degree of dissolution were noted, along with approximate pH value (to allow comparisons between different extractions of the same extract). Finally, dissolved powder extracts were added to cell culture media, to create concentrations ranging between 0.1 and 10%. Each treatment solution was filtered using a 0.22 µM filter before addition to cells.

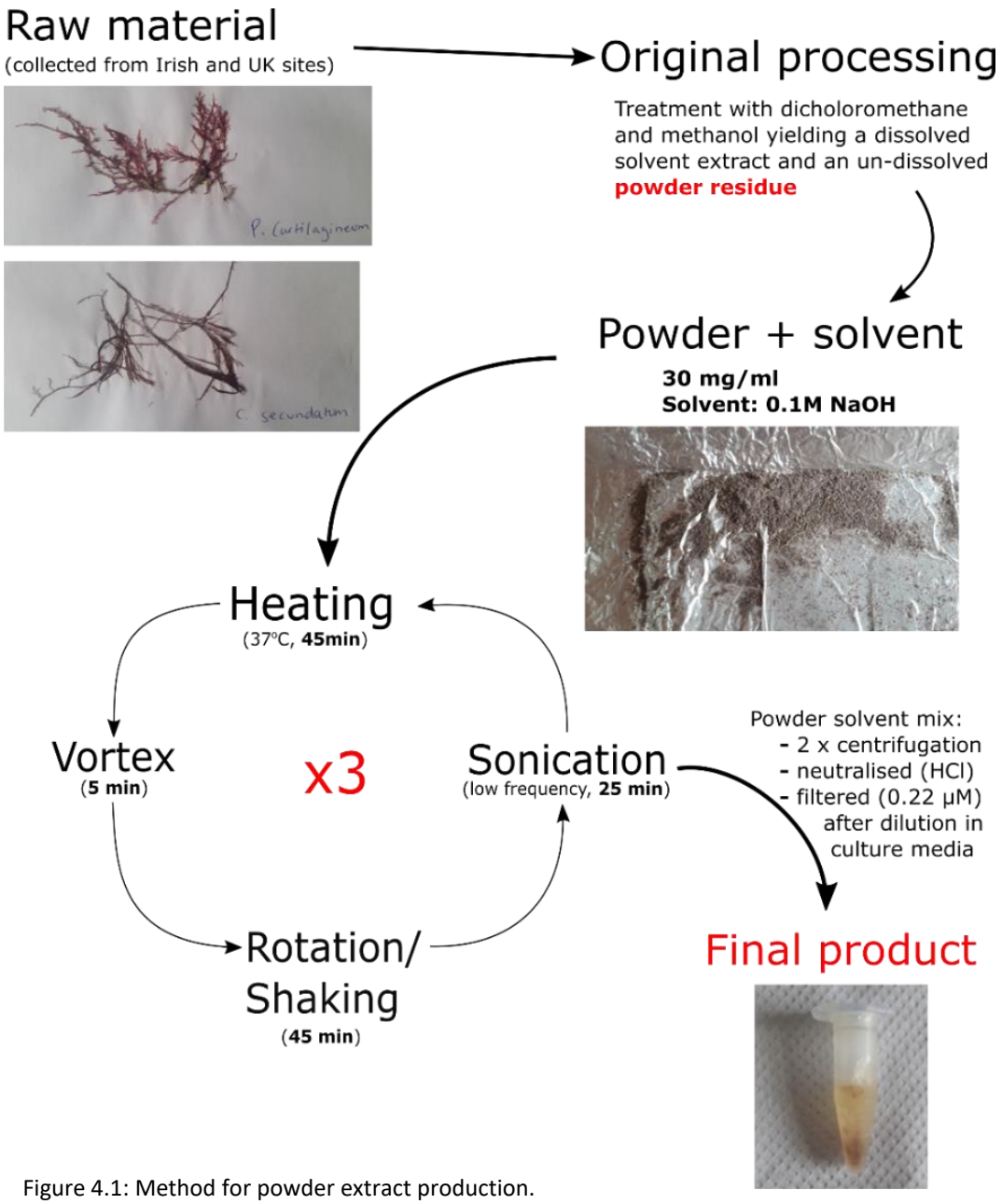


Figure 4.1: Method for powder extract production.

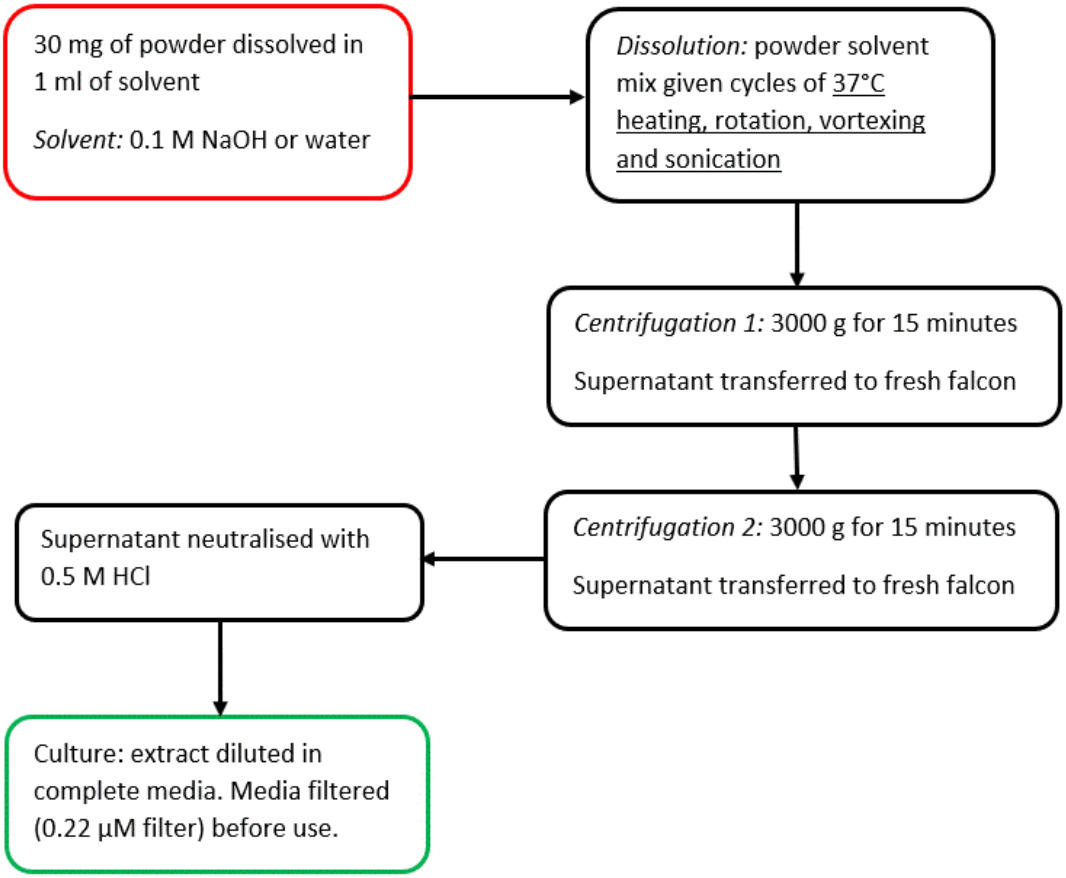


Figure 4.2: Extract production from powder residue material (left over from original organic extractions).

4.2.2.4 Converting concentrations from percentages to µg/ml

Treatments containing powder extracts were originally produced so that culture media contained a stated percentage of extract solution. To convert these percentages to a more informative µg/ml value a 500 µl aliquot was placed into a pre-weighed 1.5 ml Eppendorf, with 5 holes punctured through the cap using a needle. Each Eppendorf was then frozen (-80°C) and placed in a freeze dryer (Christ Alpha RVC vacuum centrifuge coupled to a Christ Alpha 1-4 freeze dryer, Germany) for 24 h (settings: -45°C, 10 mbar pressure, 300 g). After drying, each Eppendorf was re-weighed, allowing the amount of extract material contained in the 500 µl aliquot to be determined. This procedure was repeated a total of three times for each powder extract tested, allowing an average µg/µl concentration value to be determined (table 4.2); giving a better indication of the amount of organic material present in final treatments. As a known volume of extract solution was used to create each treatment

solution, this µg/µl average allowed percentage values to be converted into µg/ml. These conversion values were subsequently used in the remainder of this thesis.

Table 4.2: calculation of total powder extract weight contained within aliquots of treatment solution, after drying. For each extract solution 3 aliquots (from 3 separate extractions) were dried and weighed. Weights were converted to µg/µl concentrations, as displayed. The average concentration is also shown for each powder extract.

			µg/µl concentration			
ID number	**Genus, species**	**Solution**	**Set 1**	**Set 2**	**Set 3**	**Average**
614	*C. secundatum*	Saline	5.5	6.3	9.0	6.9
614 UK	*C. secundatum*	Saline	6.6	10.5	8.9	8.7
614 Ire	*C. secundatum*	Saline	8.6	7.2	7.7	7.8
333	*P. lyngbyanum*	Saline	5.1	8.2	6.5	6.6
334 UK	*P. cartilagineum*	Saline	7.4	7.9	10.8	8.7
334 Ire	*P. cartilagineum*	Saline	9.0	11.7	10.5	10.4
625	*Osmundea sp.*	Saline	13.8	15.9	14.6	14.7
294	*B. fruticulosa*	Saline	6.7	8.8	12.7	9.4
615	*C. pallidum*	Saline	15.5	15.2	15.2	15.3
615	*C. pallidum*	Water	15.7	15.0	15.0	15.2
621	*Punctaria sp.*	Water	7.2	7.6	7.1	7.3
840	*S. muticum*	Saline	11.3	11.1	7.6	10.0
632	*C. spongiosus*	Saline	6.6	6.3	7.2	6.7
1358	*P. squamatus*	Saline	5.3	4.9	5.5	5.2

4.2.3 General experimental design

For each of these experiments multiple controls were included, to test saline or water solvents at all concentrations appropriate for the extracts (i.e. ranging between 0.1-10% volume). All controls were compared (via one-way ANOVA) to see if there was significant difference between them. If comparable, then controls were grouped together and all extract treatments were compared to the one average control value. Alternatively, if deviation did occur then those values which were significantly different from the rest were separated, and used to determine the statistical significance of extract treatments at the same concentration.

See figure 4.3 for an overall summary of experimental design.

4.2.3.1 Screening for effects on proliferation

- Cells were seeded onto 96-well plates at a $2x10^4$ density, with 3 or 4 repeat wells per treatment. They were given a 24-hour attachment period, after which culture media was changed to that containing treatments.
- hBMSCs were from donors 002 (passage 5) and 005 (passage 4).
- Day 1, 4 and 7 time points were included on separate plates. Cells were fed with 200 µl of treatment media on day 4.
- All proliferation experiments used complete media: α-MEM media (ThermoFisher Scientific, 22561-021) containing FBS (5 or 10% FBS, depending upon the growth rate observed), 2mM L-glutamine and 100 U/ml pen-strep.
- Day 1, 4 and 7 crystal violet assays were performed using cell monolayers. 1 M acidified methanol (100 µl) was used to resolubilise crystal violet stain solution and a blank was included on each test plate, to account for background absorbance.

4.2.3.2 Screening for effects on differentiation

- Cells were seeded onto 96-well plates at a $1x10^4$ or $2x10^4$ density, with 3 or 4 repeat wells per treatment. They were given a 24-hour attachment period, after which culture media was changed to that containing treatments.
- hBMSCs were from donors 008 (passage 4-5) and 004 (passage 5). 004 cells grew more rapidly than those of 008 and were therefore included at the lower $1x10^4$ density.
- Day 7 and 14 time points were included on separate plates. Cells were fed with 200 µl of treatment media on day 4, 7 and 10.
- All differentiation experiments used osteogenic treatment media: complete media recipe (5 or 10% FBS, depending on the growth rate observed) supplemented with 50 µM ascorbate-2-phosphate and 10 µM β-glycerophosphate.
- In addition to regular vehicle controls in osteogenic media, a complete media only control was included.
- Day 7 and 14 alkaline phosphatase activity assays were performed with ALP readings normalised to DNA concentration (determined via PicoGreen assay).

4.2.3.3 Screening for effects on mineralisation

- Cells were seeded onto 24-well plates at a $2x10^4$ density, with 4 repeat wells per treatment. They were allowed to reach 75% confluency, after which culture media was changed to that containing treatments.
- hBMSCs were from donors 003 (passage 3, 5) and 006 (passage 3).
- Day 14 and 21 time points were included on separate plates. Cells were fed with 200 µl of treatment media on day 4.
- All mineralisation experiments used mineralogenic treatment media: complete media recipe (10% FBS) supplemented with 50 µg/ml ascorbate-2-phosphate and 2 M β-glycerophosphate.
- In addition to regular vehicle controls, a mineralogenic only and complete media only control was included in each layout.
- Alizarin red-S (AR-S) assay was performed, with optical density read at 550 nm and converted to a value for AR-S concentration (via standard curve).

4.2.3.4 Effects of 'in-house' extracts

- Cells were seeded onto 96-well plates at a $1x10^4$ density, with 4 repeat wells per treatment. They were given a 24-hour attachment period, after which culture media was changed to that containing treatments.
- hBMSCs were from donor 008 (passage 6).
- Day 4 and 6 time points were included on separate plates. Cells were fed with 200 µl of treatment media on day 4. Complete media was used (5% FBS).
- Treatments were included from various extractions of *C. secundatum* (614) and *P. cartilagineum* (334) 'in-house' material, including: **1**. normal powder extract material (termed 'powder extract normal'), **2.** powder extract from ethanol extraction (termed 'powder extract ethanol'), **3.** DCM/MeOH extraction reconstituted in ethanol and **4.** Ethanol extraction reconstituted in ethanol.
- Day 4 and 6 crystal violet assays were performed as previously detailed.

4.2.3.5 Subfraction testing

- Experimental conditions and assays used were the same as that of 4.2.3.1, using hBMSCs from donor 001 (passage 4).

- Extract solutions contained material processed first by ultrafiltration, using Amicon Ultra-0.5 Centrifugal Filter Devices with a 3000 Nominal Molecular Weight Limit (NMWL). 0.5 ml of powder extract solution was centrifuged at 14,000 x g for 5 minutes, producing both filtered (<3000 NMWL portion) and retained material portions (>3000 NMWL portion).

4.2.7 Statistical analysis

Results are presented as means +/- standard deviation, based on at least 3 single repeat values for each data point. Each dataset was then tested for normality, using both Kolmogorov-Smirnov and Shapiro-Wilk tests, as well as for equal variance using Brown-Forsythe test. Subsequently, sets were tested by One-Way ANOVA, with any significant differences further investigated by Dunnetts t-test (two-sided). This compared each treatment to the medium and vehicle control group. 2 sets of results did not have equal variance and were therefore analysed by Kruskal-Wallis test instead. These included results shown in figures 4.11 a) and 4.15.

One change has been made to the statistical analysis method from that of chapter 3. Now, when comparing treatments to control, different levels of significance are included for each comparison. This is depicted using stars, which have the following meaning: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Furthermore, a difference between treatment and control will only be deemed statistically significant with p values of 0.01 or below (i.e. two or more *). This is because, based on the screening nature of this work, many statistical comparisons had to be made, increasing the likelihood of a type one error. Use of a lower p value reduces this risk and makes any conclusions drawn more robust (Senn 2001).

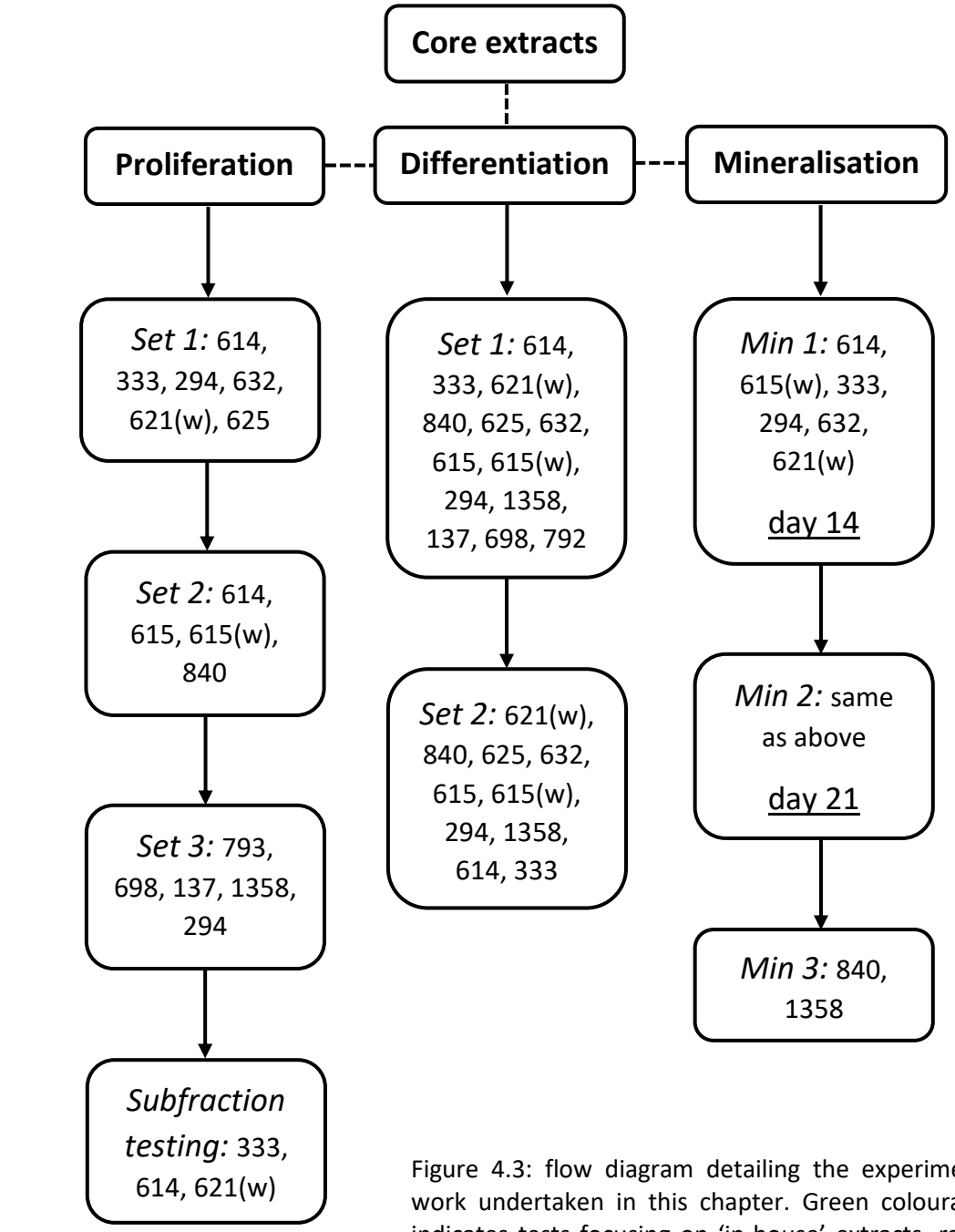
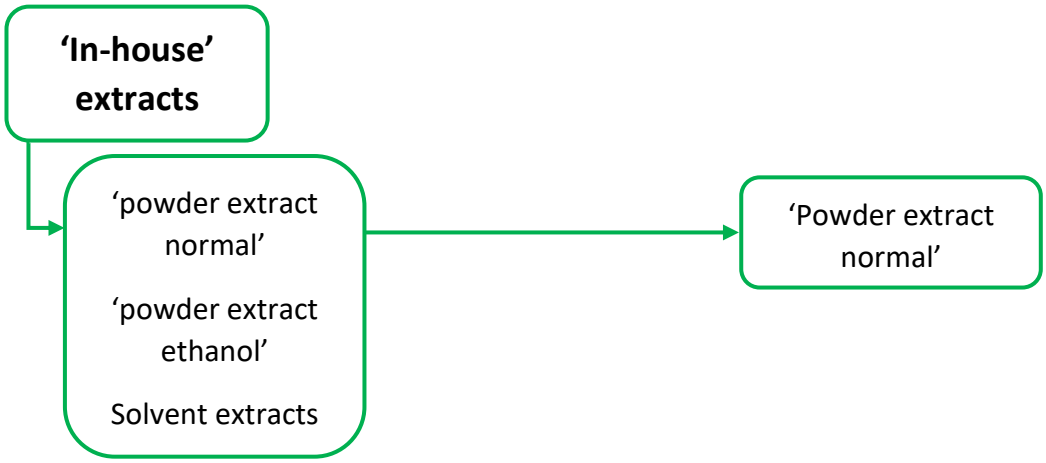


Figure 4.3: flow diagram detailing the experimental work undertaken in this chapter. Green colouration indicates tests focusing on 'in-house' extracts, rather than core supplied ones.



4.3 Results

4.3.1 Screening for effects on proliferation

Before conducting a full screen for proliferative effects of powder extracts a smaller preliminary test was performed (results not shown), to ensure that undissolved material was no longer a factor affecting CV assays. Fortunately, use of two centrifugation cycles coupled with filtration of treatment media eliminated the non-specific staining issue (as depicted by images of monolayers in figure 4.5). After this initial testing, hBMSC proliferation was determined for each powder extract. This involved testing a comprehensive range of 5 concentrations (ranging from very low to very high µg/ml values) for new extracts and a smaller subset of promising concentrations for previously tested samples; as determined during hFOB preliminary testing (see chapter 3). Due to the number of extracts tested they have been presented in three separate sets.

For the first set at day 1 [figure 4.4 a)] there was generally little deviation in extract treatment values from controls. One major exception to this was 621(w), which caused a large and highly significant increase in cell number at both concentrations tested. Alternatively, the second extraction showed a lower increase, but was still significant at 730(2) µg/ml concentration. 333 also caused a small increase in cell number at the 8 µg/ml treatment, whilst 632 appeared to dose-dependently decrease proliferation. By day 4, 317 µg/ml 632 significantly reduced proliferation [4.4 b)], whilst 333's proliferative effect was more pronounced for all treatments, causing an approximate 3000-4000 increase in cell number - which peaked at 40 µg/ml. At this time point 614 and both extractions of 621 also caused significant proliferation increases, with 614 similar in size to 333 whilst 621(w) again showed the greatest effect on proliferation. By day 7 [4.4 c)] 614 and 333's proliferative effects had grown more significant. In particular, 333 caused substantial hBMSC growth promotion, resulting in an increase of over 9000 cells compared to the saline control. 621(w) was more variable, with the second extraction increasing proliferation less than the first and only reaching significance at 730(2) µg/ml. Contrary to these effects, extract 632 still showed lower cell number values than control, though these reductions were no longer significant. Across all time points both extractions of 625 had no significant effect on cell proliferation, and were similar to control.

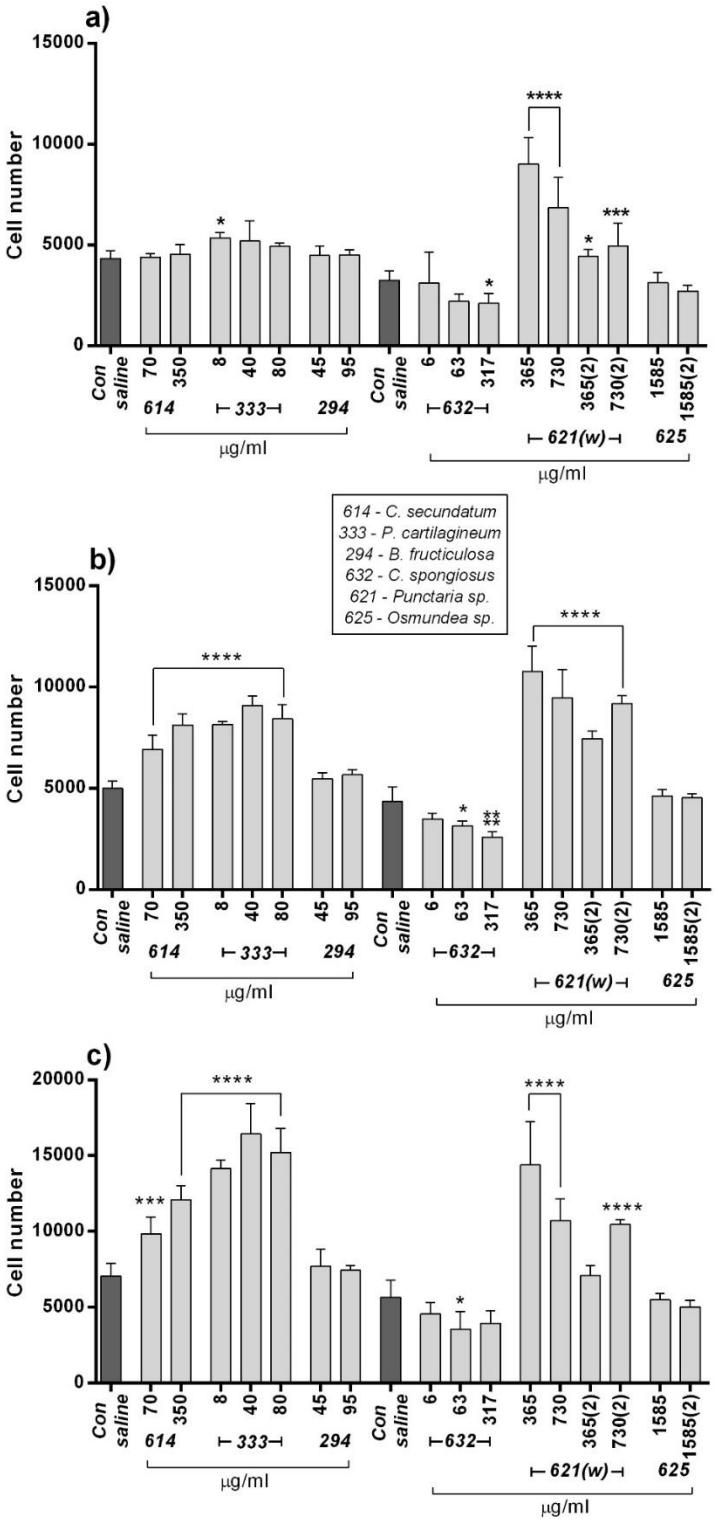


Figure 4.4: results from two separate tests of hBMSC (donor:002, passage:5 on the left and donor:005, passage:4 on the right) proliferation at day 1 [a)], 4 [b)] and 7 [c)] using crystal violet assay. Control treatments included complete media with saline solution between 0.1-10% (Con saline). A separate control is shown for each test. Cells were also challenged with extracts 614, 333, 294, 632, 621(w) or 625 at varying concentrations, dissolved in either saline solution or water (w). (2) indicates a separate extraction from the same sample material. Cell number is presented as mean +/- SD, (n=3). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Cell monolayers were also stained with crystal violet and imaged (figure 4.5) after 7 days of extract treatment. These confirm that treatments had no evident toxic effect and that cell number and degree of staining are increased with extract treatment – especially 333 and 621(w).

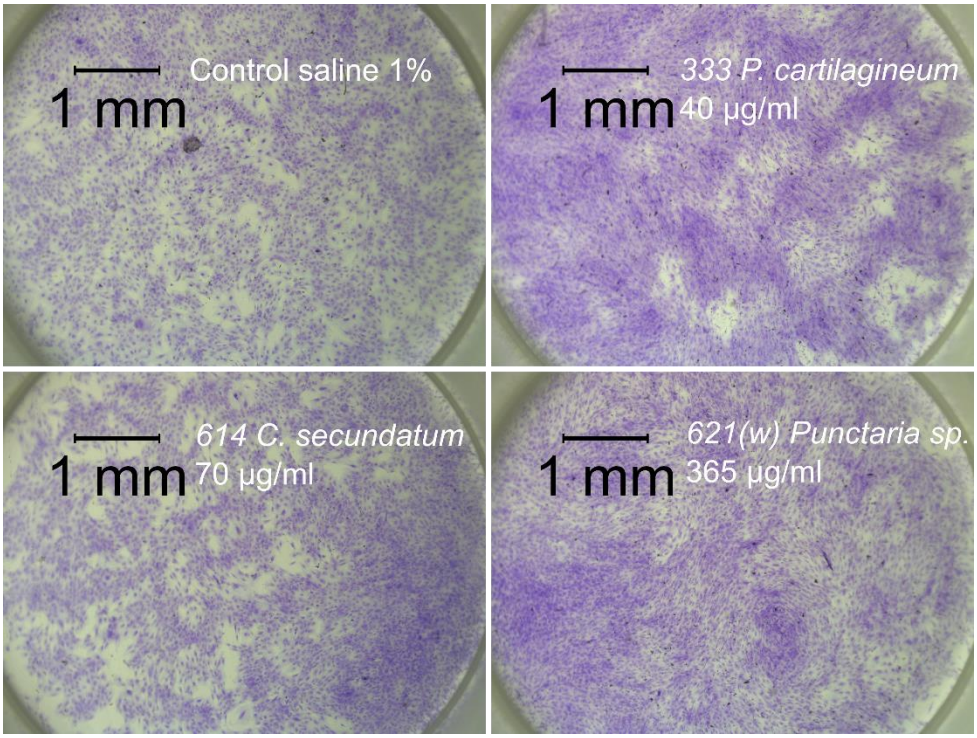


Figure 4.5: Day 7 hBMSCs (donor: 002, passage:5) challenged with either a control (saline 1%) or extract treatments 333, 614 and 621 at varying concentrations. Images show the whole of one treatment well, from a 96 well plate, stained with crystal violet.

For set 2, extract treatments showed larger deviations at day 1 [figure 4.6 a)], with 614 resulting in significantly increased cell number at both concentrations - but peaking at 350 µg/ml (average cell number: 7534, control: 5099). Alternatively, 615 caused a significant and dose dependent reduction in cell number at all concentrations excluding the 15 µg/ml treatment. 615(w) and 840 also followed this trend, though only their highest treatment concentrations - 1520 and 500 µg/ml respectively, reached significance. At day 4 [4.6 b)], cell

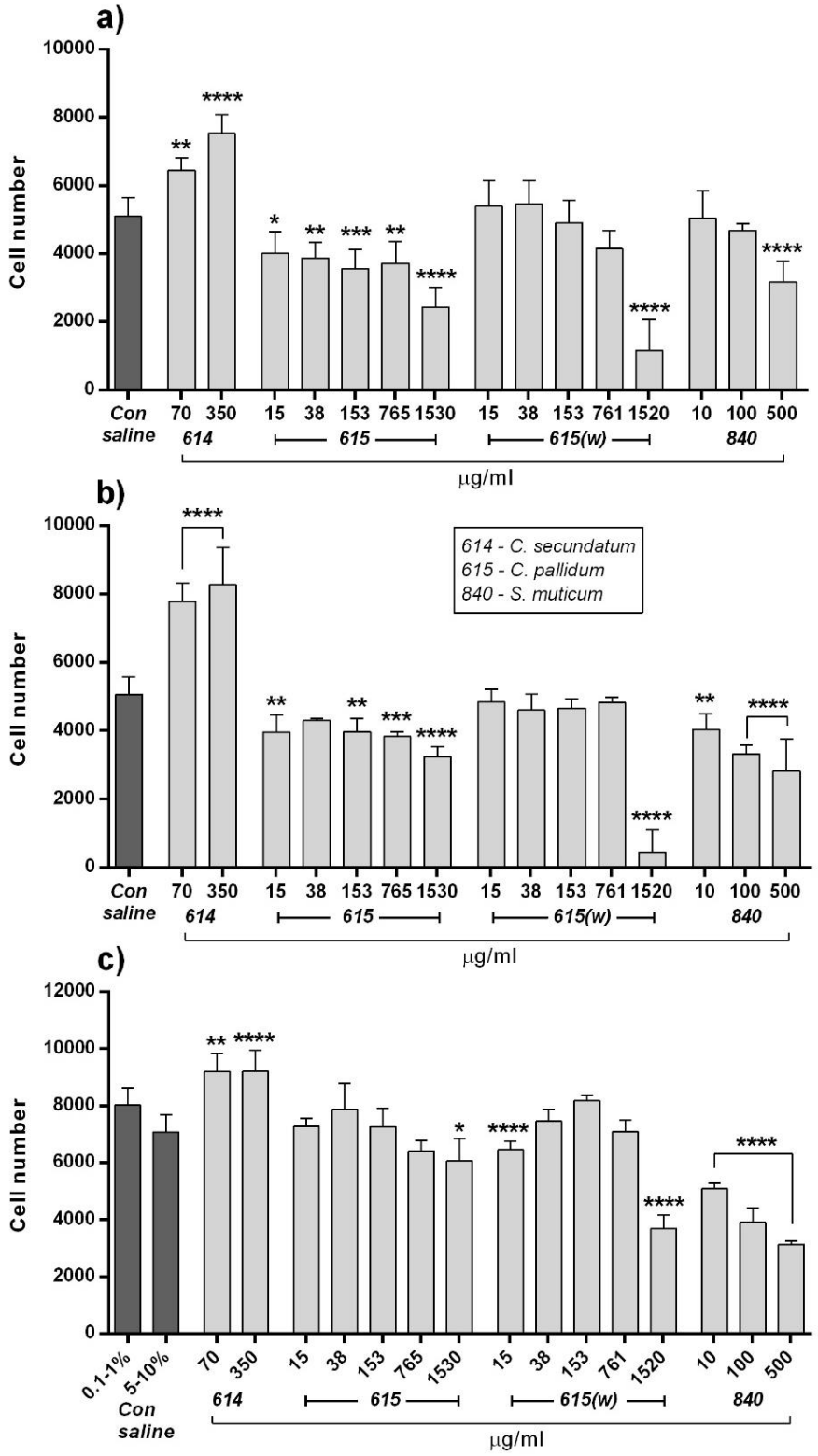


Figure 4.6: hBMSC (donor:005, passage:4) proliferation at day 1 [a)], 4 [b)] and 7 [c)] using crystal violet assay. Control treatment was complete media with saline solution 0.1-10% (Con saline). Cells were also challenged with extracts 614, 615, 615(w) and 840 at varying concentrations. Cell number is presented as mean +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

number trends remained similar with some small variation. For example, cell number increases with 614 treatment were more pronounced, reductions with 615 were slightly reduced and the 38 µg/ml treatment was no longer significant. For 615(w) a dose-dependent increase was no longer apparent, though cell number was still very low for the 1520 µg/ml treatment (average cell number: 454, control: 5065). 840 retained its dose dependent effect, with all treatments now being significantly reduced in cell number compared to control. At day 7 [4.6 c)], 614 and 840 retained similar effects, though 614's stimulative impact was slightly decreased. On the other hand, 615 and 615(w) displayed a bell-shaped response, with cell number peaking at 38 µg/ml for 615 and 153 µg/ml for 615(w). For both treatment groups the highest concentrations still reduced proliferation, though this was only significant for 615(w), as was the reduction with 15 µg/ml of this treatment.

Set 3 of the cell proliferation screen showed much less variation in cell number at day 1 [figure 4.7 a)]. Most treatments were similar in value to control, and whilst some increases and decreases in cell number were apparent none of these were significant. By day 4 [4.7 b)], clearer trends were apparent – such as an increase in cell number with 793 treatment. 698 showed a dose dependent reduction, as did extract 137, whilst all concentrations of extract 1358 caused cell number reductions – though only the 510 µg/ml treatment was significant. Contrary to its previous effect, extract 294 caused a dose dependent decrease in cell number. Day 7 [4.7 c)] results were like those of day 4, with 793 promoting cell number to a significant degree – excluding the 1% treatment. Similarly, 1358 still significantly reduced proliferation at 510 µg/ml, though 50 and 255 µg/ml treatments no longer caused reductions. Extract 294 also maintained its dose dependent reduction of cell number, showing a significantly increased cell number at 10 µg/ml (average cell number: 9433, control: 8064) and significantly reduced at 470 µg/ml (average cell number: 4995). However, extracts 698 and 137 varied slightly from their day 4 trend, showing smaller deviations from the control value (though as before no significant effects were seen).

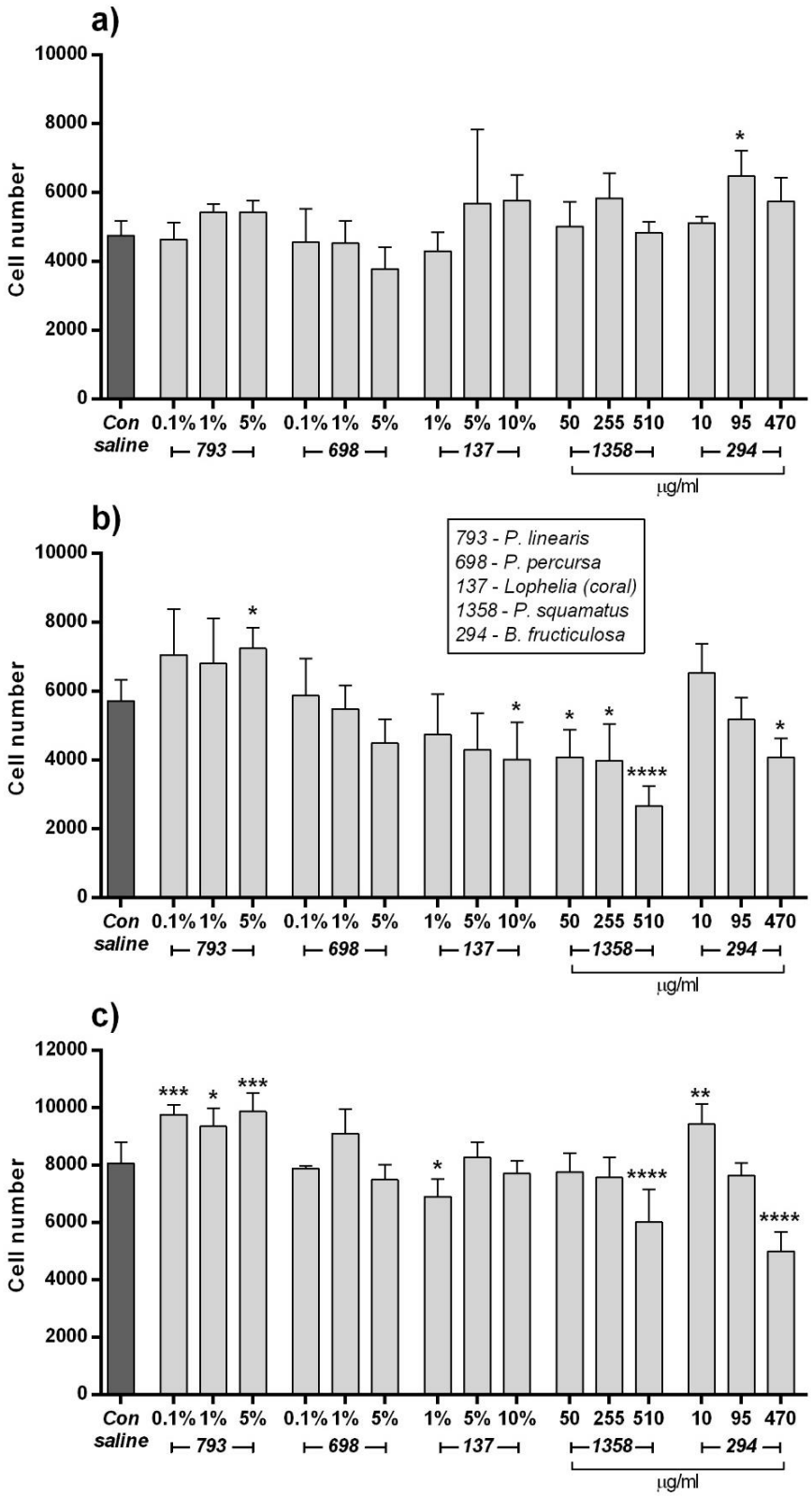


Figure 4.7: hBMSC (donor:005, passage:4) proliferation at day 1 [a)], 4 [b)] and 7 [c)] using crystal violet assay. Control treatment was complete media with saline solution at 1, 5 or 10% (Con saline). Cells were also challenged with extracts 793, 698, 137, 1358 or 294 at varying concentrations. Cell number is presented as mean +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

4.3.2 Screening for effects on differentiation

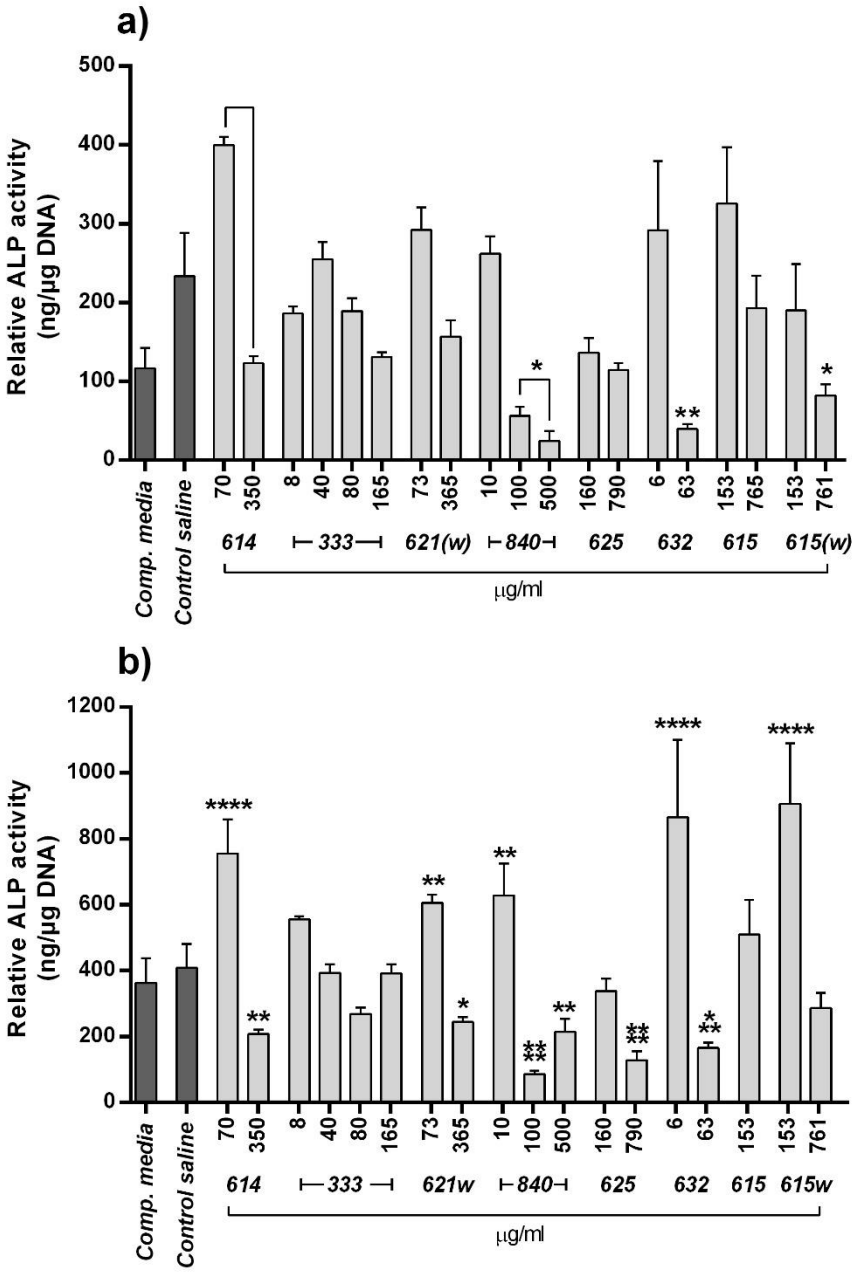


Figure 4.8: **set 1, layout 1;** hBMSC (donor:008, passage:4/5) differentiation at day 7 [a)] and 14 [b)] using ALP activity assay. Control treatment was osteogenic media with saline solution 0.1-10% (Control saline), along with a complete media treatment for reference. Cells were also challenged with extracts 614, 333, 621, 840, 625, 632 and 615 at varying concentrations. Cell differentiation is reported as ALP activity normalised to DNA concentration. Relative ALP activity is presented as mean +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

In addition to screening for proliferative effects, extracts were also tested for their differentiation potential – via alkaline phosphatase (ALP) activity assay. Promising concentrations of each extract were split between two separate experimental sets for clarity. Day 7 results for set 1 [figure 4.8 a)] showed that complete media promoted differentiation to a lesser degree than control saline, which consisted of media with saline solution and osteogenic supplements. However, by day 14 there was no evident difference between ALP activity levels of either control. Extract 614 at 70 µg/ml concentration was one of two treatments, the other being 615 – 153 µg/ml, which resulted in increases to hBMSC ALP activity at day 7. Other treatments also appeared to promote activity but not to a significant degree, such as 73 µg/ml 621(w), 10 µg/ml 840 and 6 µg/ml 632. Remaining day 7 treatments – mostly higher concentrations of those extracts previously stated – tended to decrease ALP activity. By day 14 [figure 4.8 b)] most extract activity trends remained similar. Overall, almost all extracts significantly stimulated ALP activity at their lowest concentrations, whilst higher concentrations tended to decrease activity. For example, 70 µg/ml 614 still significantly promoted relative ALP activity (755, control: 408), whilst 73 µg/ml 621(w), 10 µg/ml 840 and 6 µg/ml 632 had now reached statistical significance. The main difference from the day 7 dataset was for extracts 615 and 615(w); as 153 µg/ml 615 no longer significantly promoted ALP expression, whilst cells exposed to 765 µg/ml 615 dis-attached during culturing/assay preparation (explaining their absence in the figure).

Set 1 results are continued (layout 2) in figure 4.9, where control solutions had very similar effects to those of layout 1, with control saline promoting ALP activity compared to plain media at day 7, but not at day 14. For day 7, 294 significantly promoted cell differentiation at 10 and 95 µg/ml concentrations, though 470 µg/ml treatment slightly reduced activity. This trend was broadly maintained at day 14, though increases were lower – with the 95 µg/ml treatment losing significance. For 1358, 50 and 255 µg/ml treatments significantly increased cell differentiation at day 7, whilst the 5 µg/ml concentration was no different than control. By day 14 255 µg/ml treatment had lost its activity, though 50 µg/ml still substantially promoted ALP expression (997, control: 353). Extracts 137 and 792 had similar activity profiles as each other, causing slight reductions in ALP expression that were significant for the 5% concentration of 792 at day 7. Finally, extract 698 showed most activity at the 1% concentration, resulting in significant increases at both time points. Conversely, 698 5% treatment was more variable, changing from significantly increasing ALP activity at day 7 to decreasing (non-significant) it at day 14.

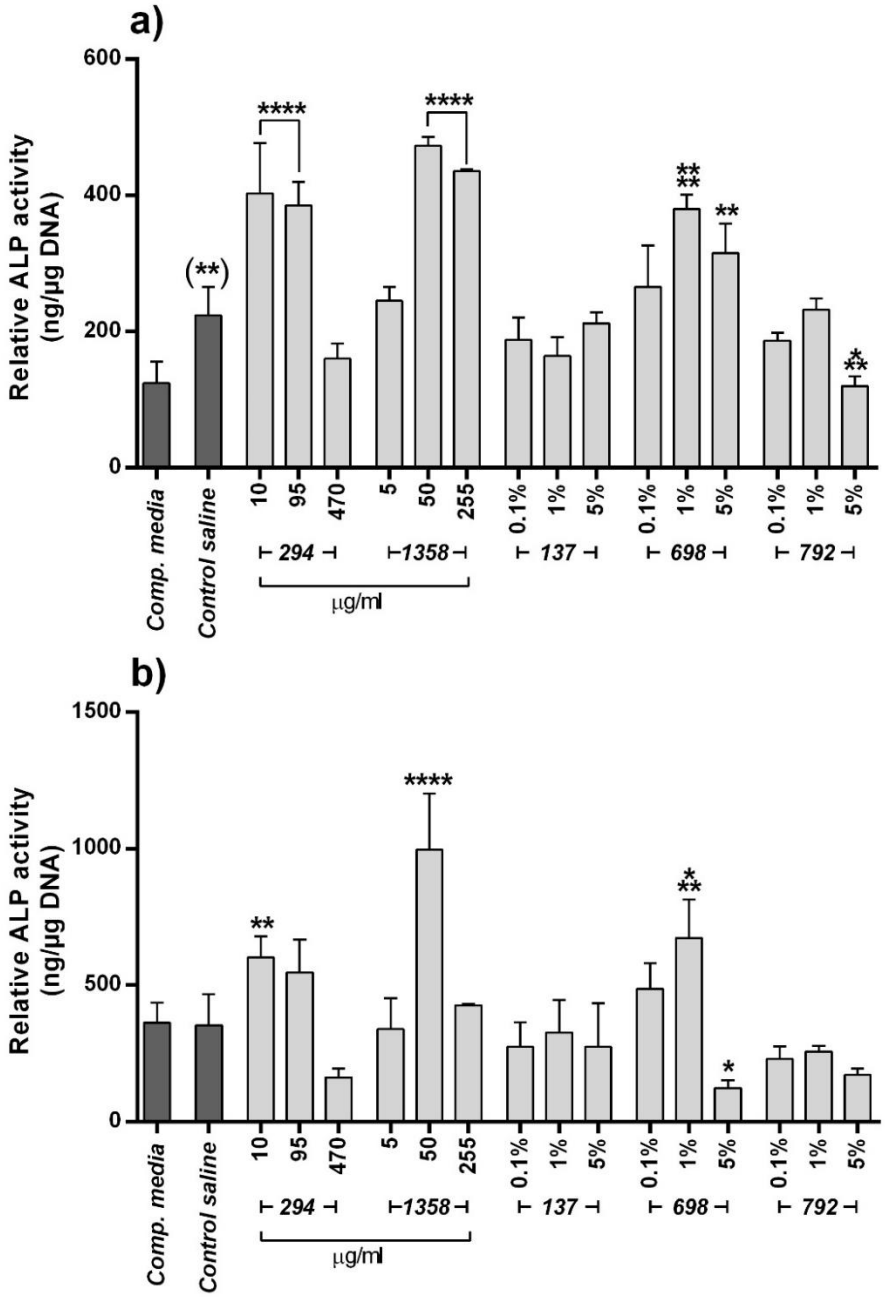


Figure 4.9: **set 1, layout 2;** hBMSC (donor:008, passage:4/5) differentiation at day 7 [a)] and 14 [b)] using ALP activity assay. Control treatment was osteogenic media with saline solution 0.1-10% (Control saline), along with a complete media treatment for reference. Cells were also challenged with extracts 294, 1358, 137, 698 and 792 at varying concentrations. Cell differentiation is reported as ALP activity normalised to DNA concentration. Relative ALP activity is presented as mean +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

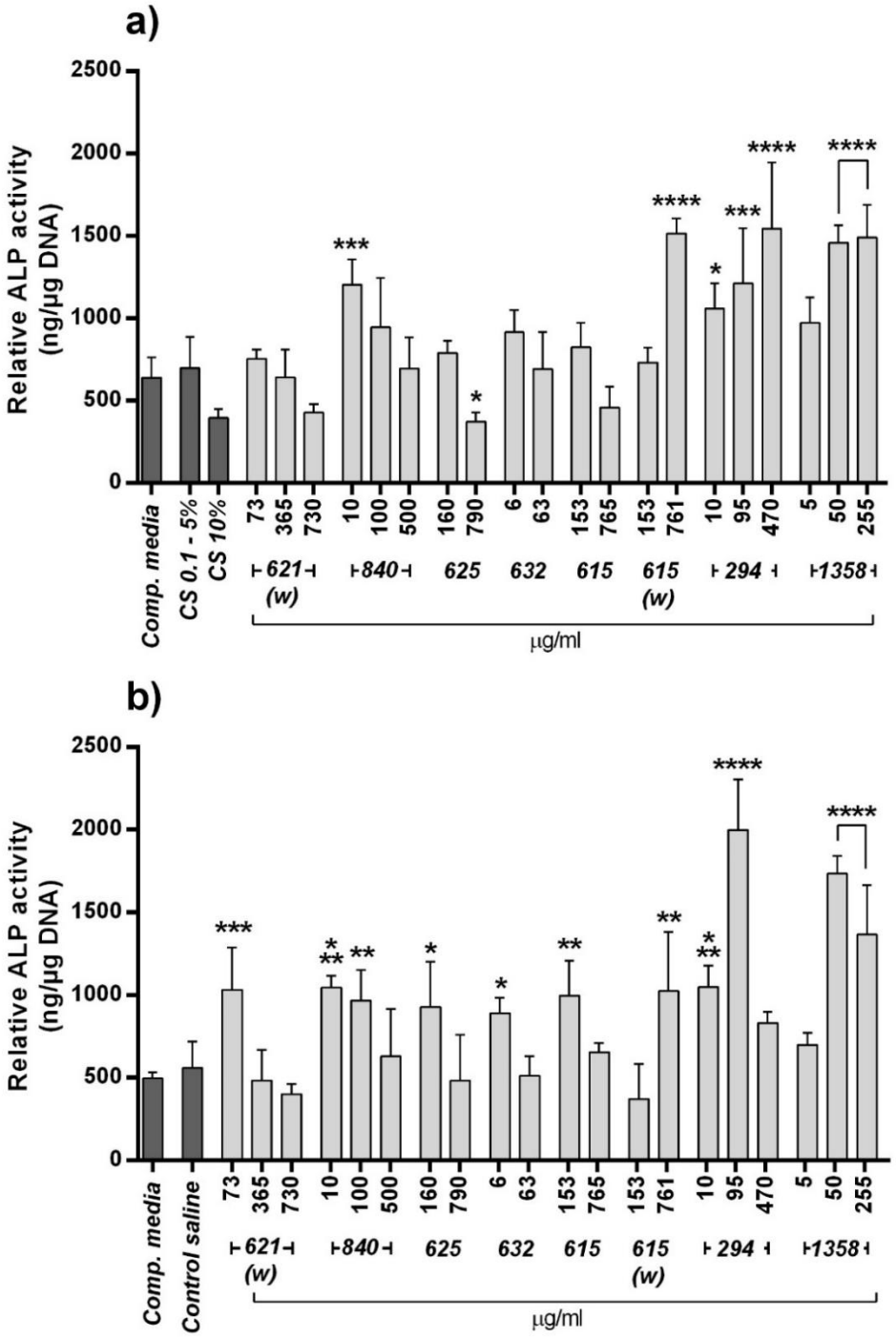


Figure 4.10: **set 2, layout 1;** hBMSC (donor:004, passage:5) differentiation at day 7 (a) and 14 (b) using ALP activity assay. Control treatment was osteogenic media with saline solution 0.1-10% (Control saline), along with a complete media treatment for reference. Cells were also challenged with extracts 621(w), 840, 625, 632, 615, 615(w), 294 and 1358 at varying concentrations. Cell differentiation is reported as ALP activity normalised to DNA concentration. Relative ALP activity is presented as mean +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

For set 2 at day 7 [figure 4.10 a)], complete media and 0.1 - 5% control saline (CS) treatments were very similar in value. Alternatively, 10% control saline treatment was much lower in value (CS 10%: 397, CS 0.1-5%: 698), being statistically different from treatments between 0.1 – 5% concentration, and was thus included separately. At days 7 and 14, extract 621(w) increased ALP activity the most at its lowest concentration – 73 µg/ml, which caused a significant promotion of activity at day 14. Higher concentrations of 365 and 730 µg/ml treatments reduced activity in a dose-dependent manner, though these reductions did not reach significance. 840 showed a similar trend, with 10 µg/ml treatment causing a significant increase at day 7 – whilst both 10 and 100 µg/ml were significant at day 14. Extracts 625, 632 and 615 also showed greatest ALP activity at their lowest test concentrations (two concentrations tested for each – low and high). On the other hand, 761 µg/ml 615(w) significantly promoted ALP activity at both time points, whilst 153 µg/ml was similar or slightly reduced in value compared to control saline treatments. At day 7, extract 294 caused a dose dependent increase in ALP activity, which was significantly increased compared to control at the two highest concentrations. However, by day 14 the 470 µg/ml treatment was reduced and had lost significance, whilst ALP activity was significantly increased compared to control for 10 µg/ml treatment. Finally, extract 1358 gave a large and significant promotion of ALP activity at 50 and 255 µg/ml concentrations, whereas 5 µg/ml was much lower in value – particularly at day 14.

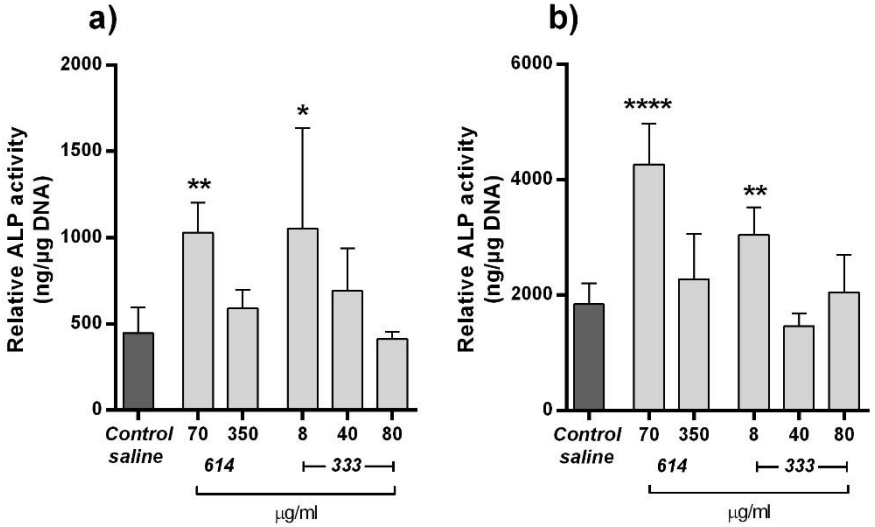


Figure 4.11: **set 2, layout 2;** hBMSC (donor:004, passage:5) differentiation at day 7 (a) and 14 (b) using ALP activity assay. Control treatment was osteogenic media with saline solution 0.1-5% (Control saline). Cells were also challenged with extract 614 at 70 and 350 µg/ml concentrations, or 333 at 8, 40 and 80 µg/ml concentrations. Cell differentiation is reported as ALP activity normalised to DNA concentration. Relative ALP activity is presented as mean +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

70 µg/ml of extract 614 significantly promoted hBMSC differentiation at both time points (figure 4.11), though the largest effect was seen at day 14. Conversely, 350 µg/ml of 614 showed no difference in ALP activity compared to control. Extract 333 also significantly promoted ALP activity levels at its lowest concentration of 8 µg/ml. This effect was of a similar size at both time points and exceeded the activity of 70 µg/ml 614 at day 7 – though the associated standard deviation was large. 40 and 80 µg/ml concentrations of 333 caused no significant change in ALP activity).

4.3.3 Screening for effects on mineralisation

For each extract tested via mineralisation assay, 4 concentrations were included. At day 14 (figure 4.12), 3 out of 6 tested extracts had little effect on hBMSC mineralisation levels, including 614, 615(w) and 632. For 614 and 615(w) treatment values were very similar to control, though 38 µg/ml 615(w) did produce a small increase in mineralisation level. 632 had no significant effect on mineralisation, but values were more variable overall – peaking at 16 µg/ml with an AR-S concentration similar to control and then decreasing at higher concentrations. Such limited mineralisation is confirmed by imaging (figure 4.13), whereby little to no AR-S staining is evident in 614 treated cells. Alternatively, extracts 333, 294 and 621(w) had obvious positive effects on hBMSC mineralisation levels, as shown qualitatively and in images of 333 and 294 treatments (figure 4.13) – where obvious AR-S staining is evident. 333 significantly increased AR-S level at all tested concentrations, though 8, 40 and 80 µg/ml treatments caused relatively small increases compared to that of 165 µg/ml 333.

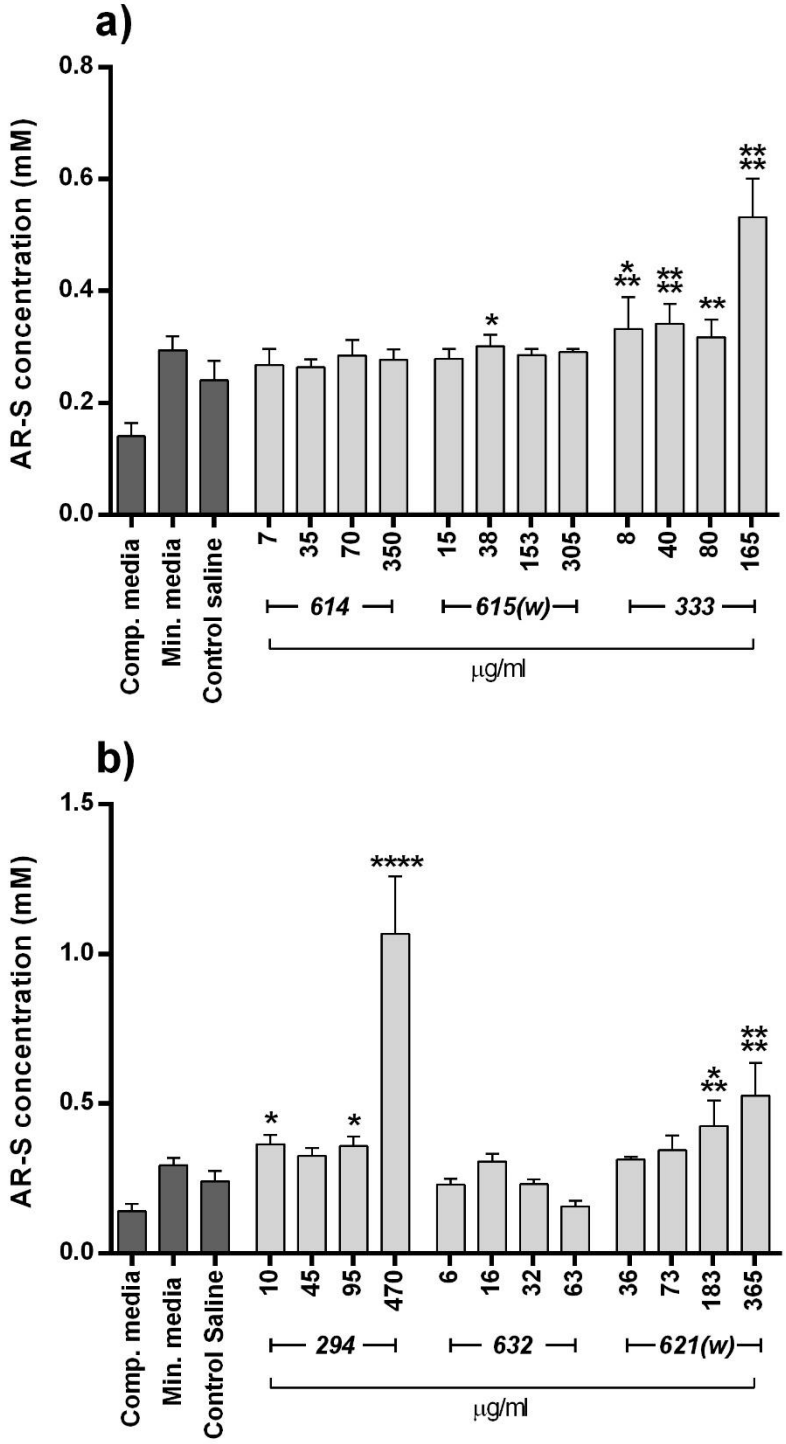


Figure 4.12: hBMSC (donor:006, passage:3) mineralisation at day 14 using alizarin red-S assay. Control treatment was mineralogenic media with 1-5% saline solution (Control Saline), though mineralogenic (Min. media) and complete media (Comp. media) only controls were also included for reference. Cells were challenged with extracts 614, 615(w), 333, 294, 632 and 621(w), with 4 concentrations tested for each. Mineralisation is presented as mean alizarin red-S (AR-S) concentration (mM), +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Extract 294 had a similar activity profile, with only small increases at 10 and 95 µg/ml concentrations – whilst the highest treatment concentration of 470 µg/ml had an obvious significant effect (AR-S concentration of 1.06, control: 0.24). Finally, 621 (w) caused a dose-dependent increase in mineralisation level which reached significance at 183 and 365 µg/ml concentrations.

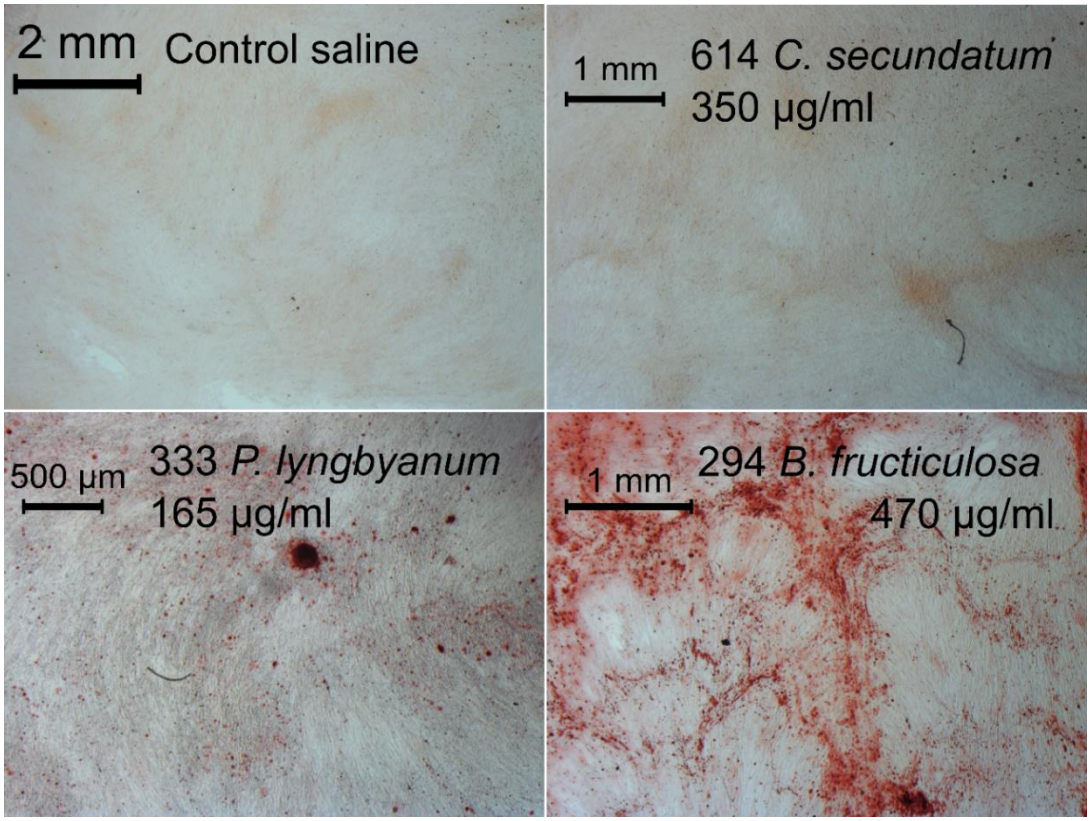


Figure 4.13: images of hBMSC (donor:006, passage:3) mineralisation at day 14, challenged with control saline solution and extracts 614, 333 and 294 at varying concentrations. Images show portions of treatment wells (24-well plate) at different magnifications, stained with AR-S.

Each extract tested in the first day 14 mineralisation assay was also retested, this time using a day 21 time point (figure 4.15). 614 maintained a similar activity trend, causing small non-significant increases in mineralisation. Extract 632 also showed comparable activity to day 14, with all treatments similar in value to control – though 63 µg/ml again had the lowest reported AR-S concentration of the assay (0.40, control: 0.48). As before, images of control and 614 extract treatment wells (figure 4.14) confirm this limited activity, with little AR-S staining visible. Conversely, 615(w) had much greater effect on mineralisation at day 21. Extract 333 stimulated a dose-dependent increase in hBMSC mineralisation, causing an approximate 15-fold increase in AR-S concentration at 165 µg/ml (7.57, control: 0.48). 621(w) again had a dose-dependent effect which was very similar to that seen with extract 333,

causing a pronounced mineralisation increase with 365 µg/ml treatment. Finally, extract 294 increased hBMSC mineralisation at all treatment concentrations, see figure 4.14 for image). Despite many treatments having much larger mineralisation levels compared to control, no increases were statistically significant. This is likely due to the statistical analysis used, as this dataset did not have equal variances and therefore had to be analysed using the Kruskal-Wallis test, as opposed to One-Way ANOVA.

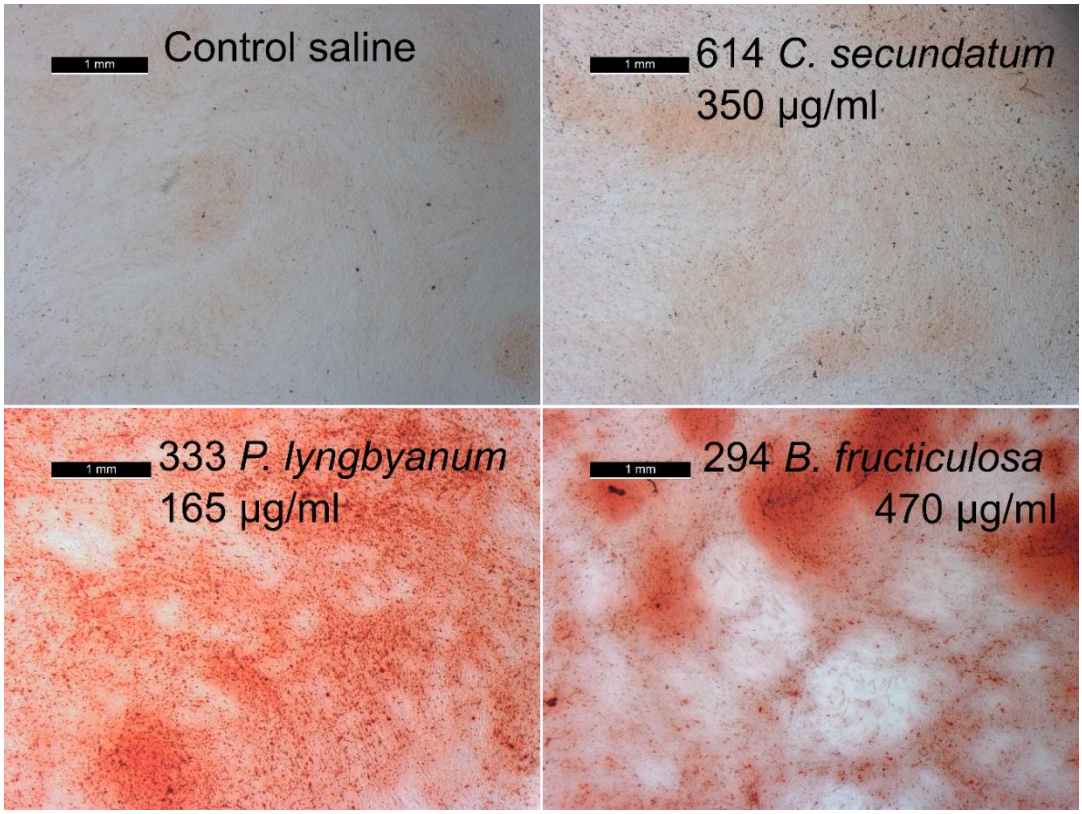


Figure 4.14: images of hBMSC (donor:006, passage:3) mineralisation at day 21, challenged with control saline solution and extracts 614, 333 and 294 at varying concentrations. Images show portions of treatment wells (24-well plate) at different magnifications, stained with AR-S.

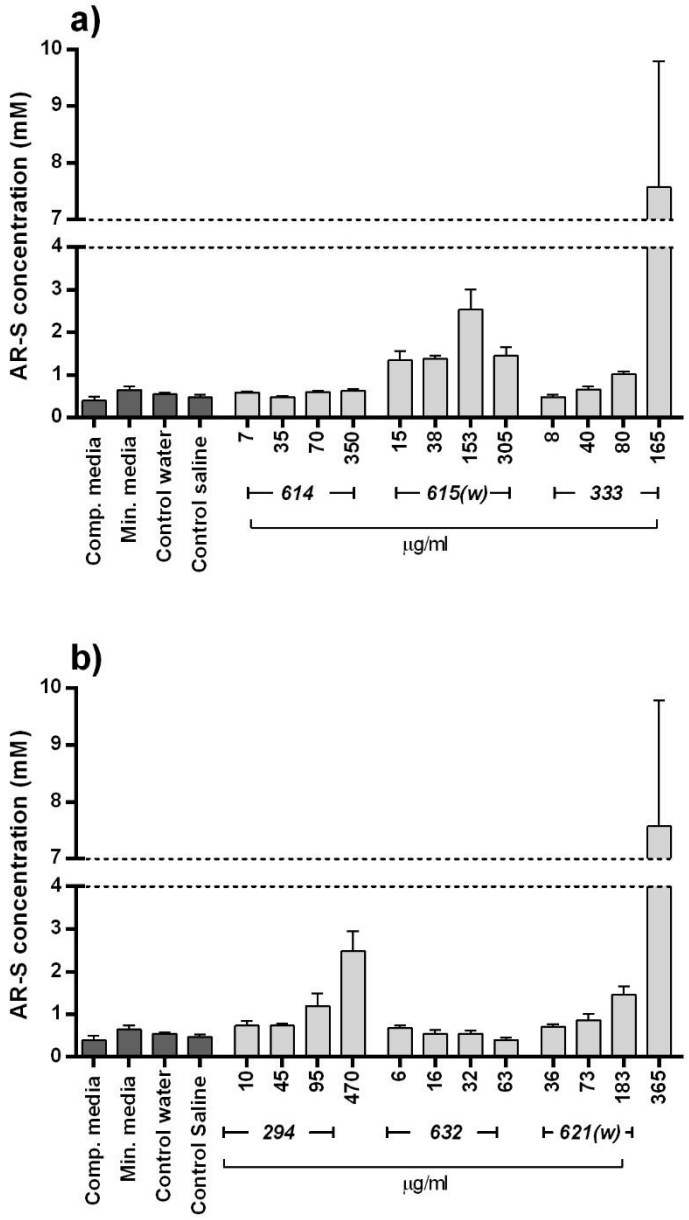


Figure 4.15: hBMSC (donor:003, passage:5) mineralisation at day 21 using alizarin red-S assay. Results are split for convenience between a) and b). Control treatment was mineralogenic media with 0.1 - 5% saline solution or 1/5% water, though mineralogenic (Min. media) and complete media (Comp. media) only controls were also included for reference. Cells were also challenged with extracts 614, 615(w), 333, 294, 632 and 621(w), with 4 concentrations tested for each. Mineralisation is presented as mean alizarin red-S (AR-S) concentration (mM), +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

A smaller mineralisation assay testing extracts 840 and 1358 was conducted at day 21 [figure 4.16]. Extract 840 decreased cell mineralisation at all tested concentrations, though none of these were statistically significant. On the other hand, 1358 caused small mineralisation

increases at 50 and 128 µg/ml concentrations. However, it was 255 µg/ml that was most active, increasing AR-S concentration to a value of 5.35 mM; a 7-fold increase from control.

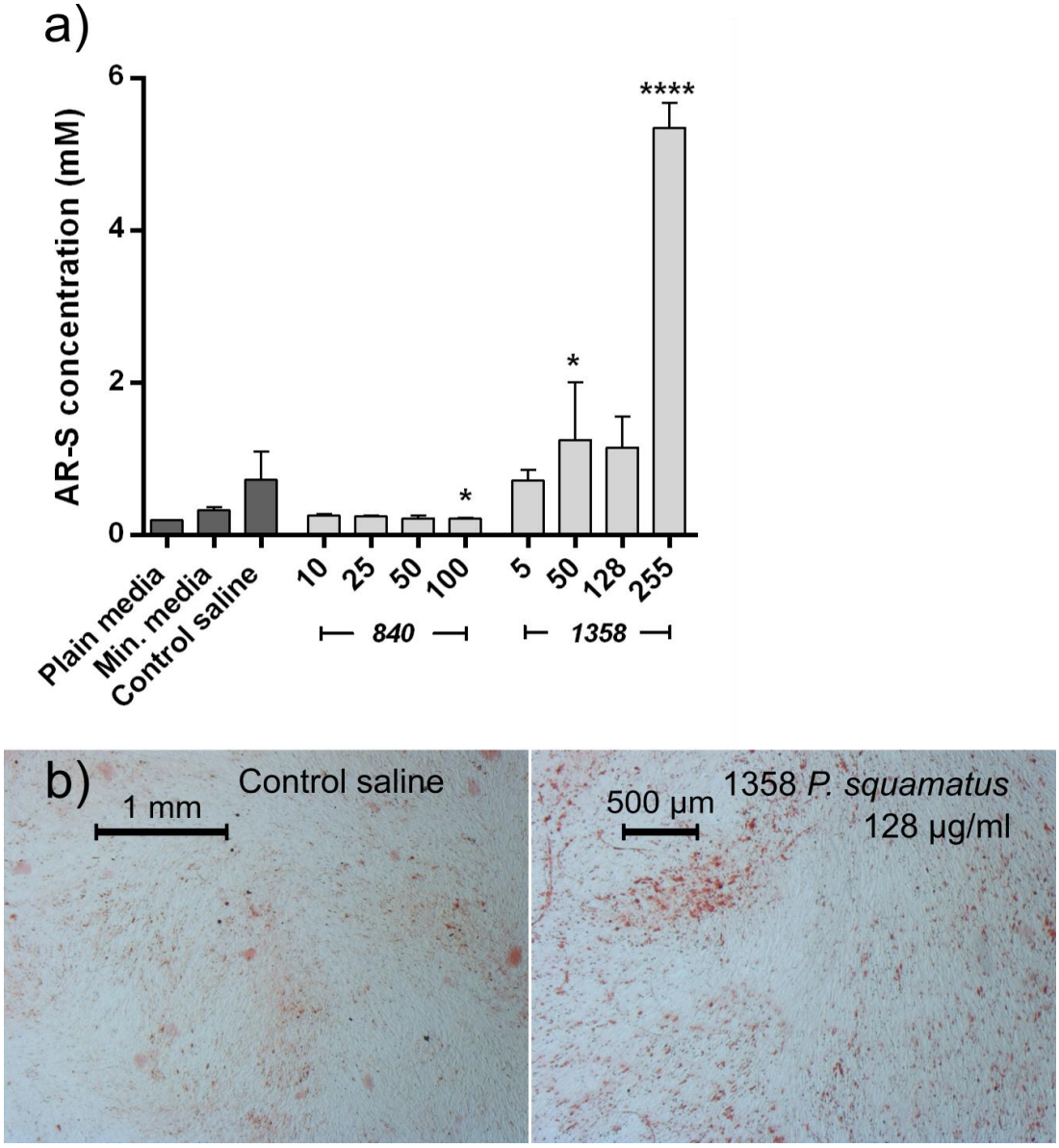


Figure 4.16: **a)** hBMSC (donor:003, passage:5) mineralisation at day 21 using alizarin red-S assay. Control treatment was mineralogenic media with 0.1 - 5% saline solution, though mineralogenic (Min. media) and complete media (Comp. media) only controls were also included for reference. Cells were also challenged with extracts 840 and 1358 [b)]. 4 concentrations tested for each extract. Mineralisation is presented as mean alizarin red-S (AR-S) concentration (mM), +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control. **b)** images of hBMSC (donor:005, passage:5) mineralisation at day 21, challenged with control saline solution or extract 1358 at 128 µg/ml concentration. Images show portions of treatment wells (24-well plate) at different magnifications, stained with AR-S.

4.3.4 Effects of 'in-house' extracts

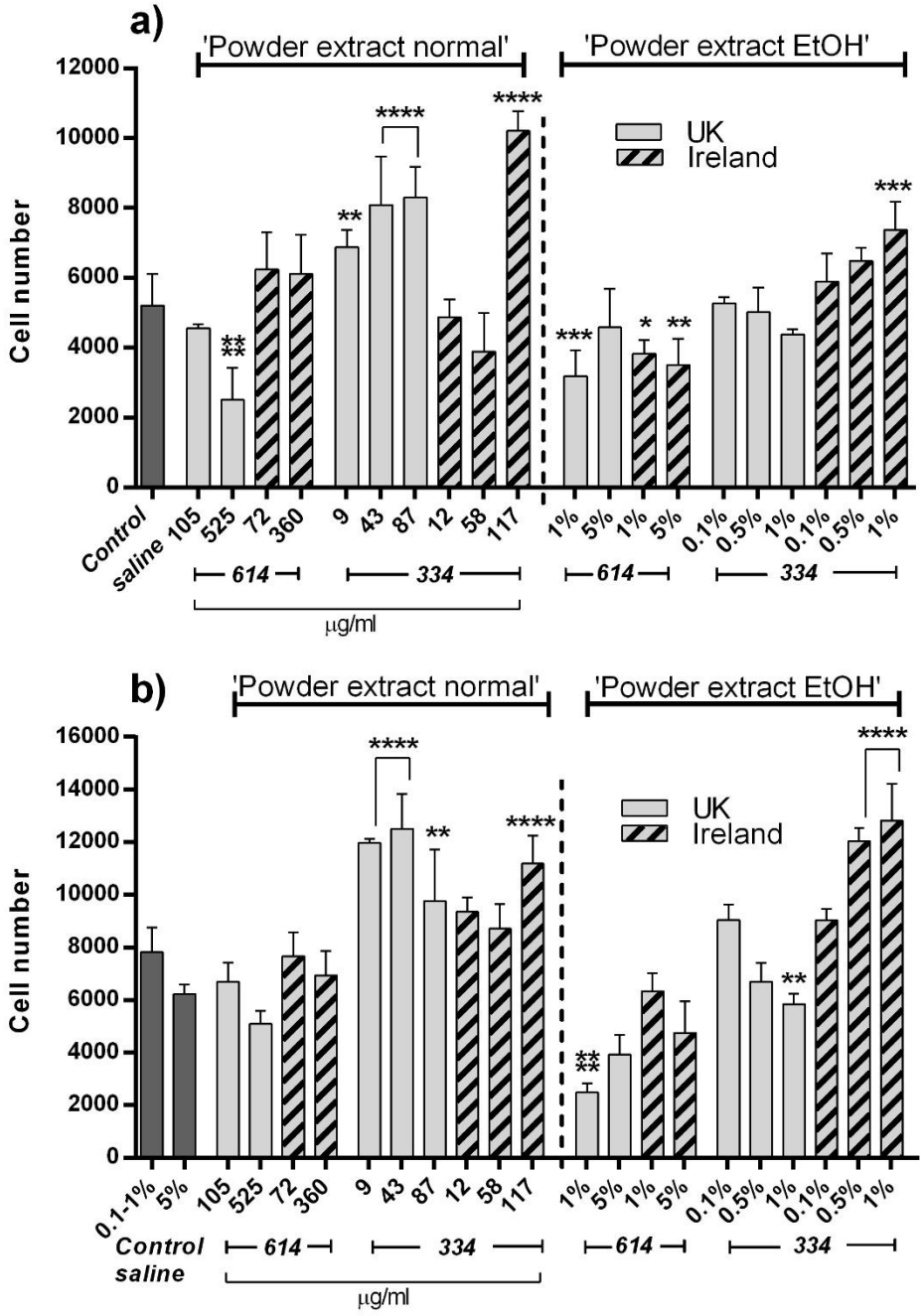


Figure 4.17: hBMSC (donor:008, passage:6) proliferation at day 4 [a)] and 7 [b)] using crystal violet assay. Control treatment was complete media with saline solution 0.1-5% (Control saline). Cells were also challenged with powder extracts *C. secundatum* (614) or *P. cartilagineum* (334) (using both UK and Irish sample site material) at varying concentrations. Powders were produced via normal (DCM/methanol) or ethanol extraction, giving two groups of treatments – 'powder extract normal' and 'powder extract ethanol'. Cell number is presented as mean +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Powder extractions performed 'in-house', using either DCM/methanol or ethanol solvents, were also tested for their proliferative effects (figure 4.17). These extractions included material from *Ceramium secundatum* (614) and *Plocamium cartilagineum* (334), collected at either a UK or Irish sample site. 'Normal' DCM/methanol extracted powders only caused a reduction in cell number at day 4 with one treatment [figure 4.17 a)] - UK 614 extract at a 525 µg/ml concentration (average cell number: 2514, control: 5203). Remaining 614 treatments from both sample locations resulted in small deviations from control values, at days 4 and 7. 'Normal' extractions of 334 on the other hand caused large sustained increases in cell number at various treatment concentrations. Irish-derived material at concentrations of 12 and 58 µg/ml are the only exceptions to this, as they did not reach significance at either time point (though at day 7 both were increased from control). Powder extractions using ethanol, 'EtOH', showed different proliferation trends than those previously described. For example, Irish-derived 334 samples promoted cell number – showing a dose-dependent effect which reached significance at day 4 for 1% treatment (average cell number: 7379, control: 5203) and at day 7 for both 0.5 and 1% treatments. Alternatively, UK-derived material had little effect on cell number, other than a significant reduction with 1% treatment at day 7 [figure 4.17 b)]. Finally, 614 treatments from both sample locations reduced cell number to varying degrees, particularly at day 4.

Overall, solvent dissolved extracts (figure 4.18) resulted in less deviation from control values than powder extracts. For example, DMSO dissolved *Ceramium secundatum* (614) and *Plocamium cartilagineum* (334) extracts had little effect on cell number at day 4, compared to control. This was also true at day 7, excluding the 0.5% concentration of 614 (Ireland) which caused a significant promotion of cell number – though it also had the largest associated standard deviation value (6229 +/- 2832 cells, control: 3978 +/- 433 cells). Ethanol dissolved extracts were very similar in their limited proliferative effect to those dissolved in DMSO, except for 0.5% 614 (Ireland). Rather than promoting cell number, as with the DMSO extract, this treatment resulted in a significant reduction at days 4 and 7.

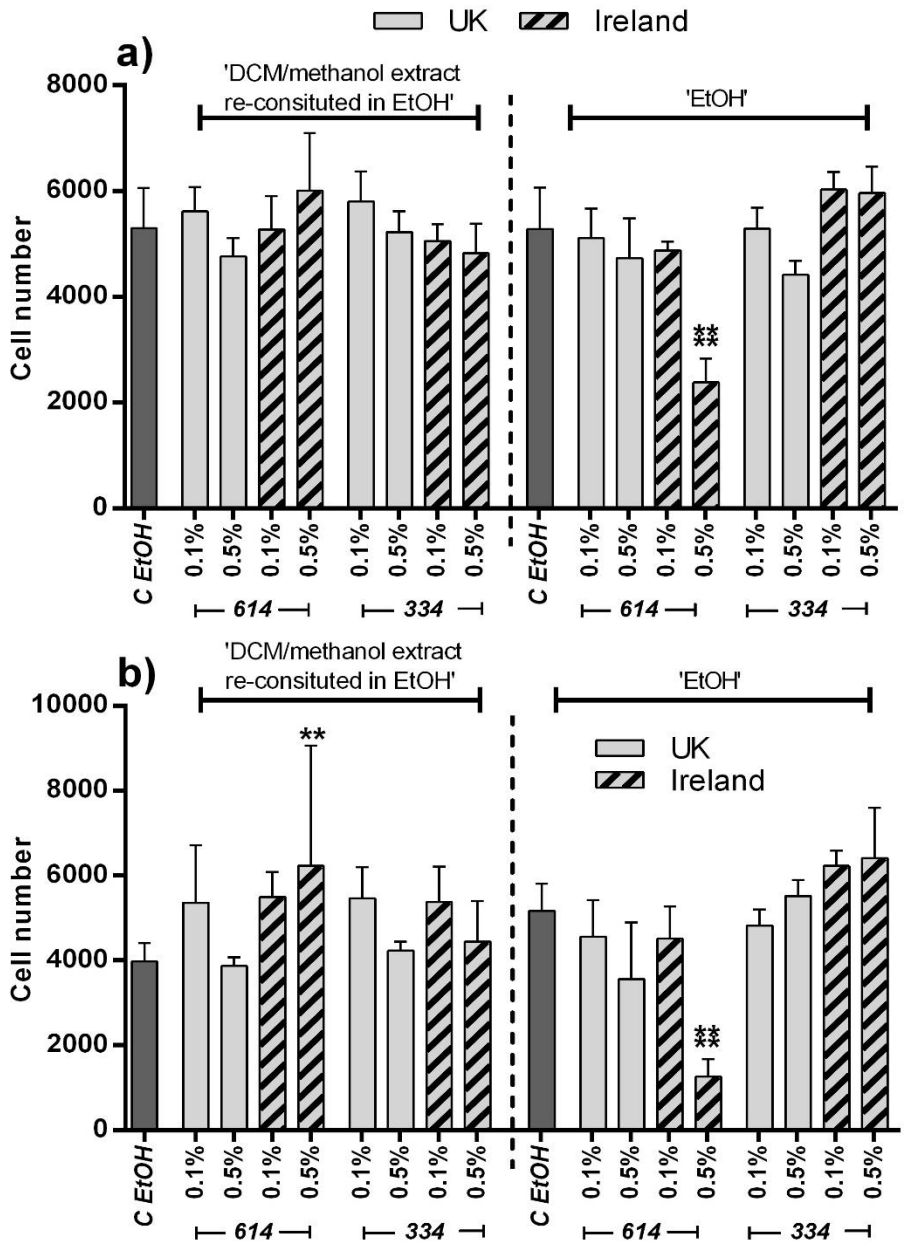


Figure 4.18: hBMSC (donor:008, passage:6) proliferation at day 4 [a)] and 7 [b)] using crystal violet assay. Control treatments included 0.1 or 0.5% ethanol. Cells were also challenged with ethanol dissolved extracts (originally extracted with either DCM/methanol or ethanol – dried extracts then re-solubilised in ethanol) *C. secundatum* (614) or *P. cartilagineum* (334) (using both UK and Irish sample site material) at either 0.1 or 0.5% concentration. Cell number is presented as mean +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

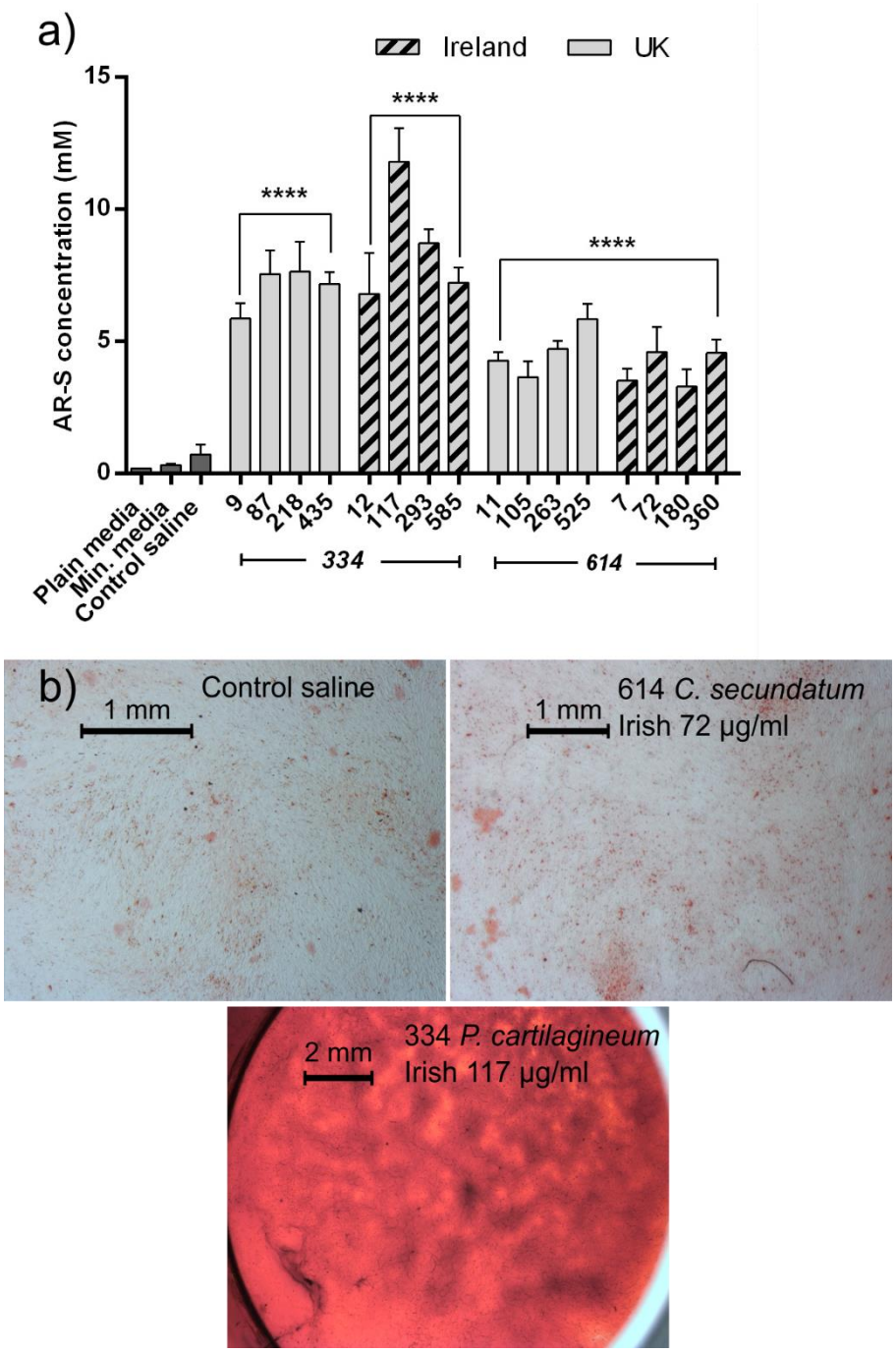


Figure 4.19: **a)** hBMSC (donor:003, passage:5) mineralisation at day 21 using alizarin red-S assay. Control treatment was mineralogenic media with 0.1 - 5% saline solution, though mineralogenic (Min. media) and complete media (Comp. media) only controls were also included for reference. Cells were also challenged with extracts 334 and 614, using sample material from both UK and Irish sample sites. 4 concentrations tested for each extract. Mineralisation is presented as mean alizarin red-S (AR-S) concentration (mM), +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control. **b)** images of hBMSC (donor:005, passage:5) mineralisation at day 21, challenged with control saline solution, extract 614 Irish at 72 µg/ml concentration or extract 334 Irish at 117 µg/ml concentration. Images show portions of treatment wells (24-well plate) at different magnifications, stained with AR-S.

In addition to CV assay, 'in-house' 614 and 334 extracts were also tested for their effects on hBMSC mineralisation. Specifically, 'powder extract normal' was tested for each species (i.e the material left over from original DCM/MeOH extraction which was then dissolved via alkaline extraction and neutralised to produce the final extract in saline solution). As in previous assays, two separate samples of *Plocamium cartilagineum* (334) and *Ceramium secundatum* (614) were included, derived from either a UK or Irish sample site [see section 4.2.2.2]. All treatments greatly promoted mineralisation at day 14, particularly sample 334 (figure 4.19 a) and confirmed by images b)] - of which most concentrations resulted in an approximate ten-fold increase in mineralisation level compared to control. Of those concentrations tested, 117 µg/ml promoted mineralisation level most, reaching an AR-S concentration of 11.81 mM – compared to a control value of 0.73. Comparatively, 614 treatments had less of an effect, though still resulted in substantial hBMSC mineralisation at all tested concentrations. Between Irish and UK samples no sustained pattern to this activity was evident, though UK material at a 525 µg/ml concentration promoted mineralisation most (5.82 mM, 8-fold increase from control).

4.3.5 Subfraction testing

Filtered (<3000 NMWL) solutions of extractions 333, 614 and 621(w) had no significant impact on hBMSC proliferation, except for the lowest concentration of 614 (350 µg/ml) which reduced cell number at days 1 and 4 (figure 4.20). However, retained material (>3000 NMWL) had a much greater impact, particularly for 333 which caused a large sustained increase in proliferation at all time points (e.g. 10252 cells at day 4, control: 4351). Retained material from extracts 614 and 621(w) caused smaller promotions, which were only significant at day 4.

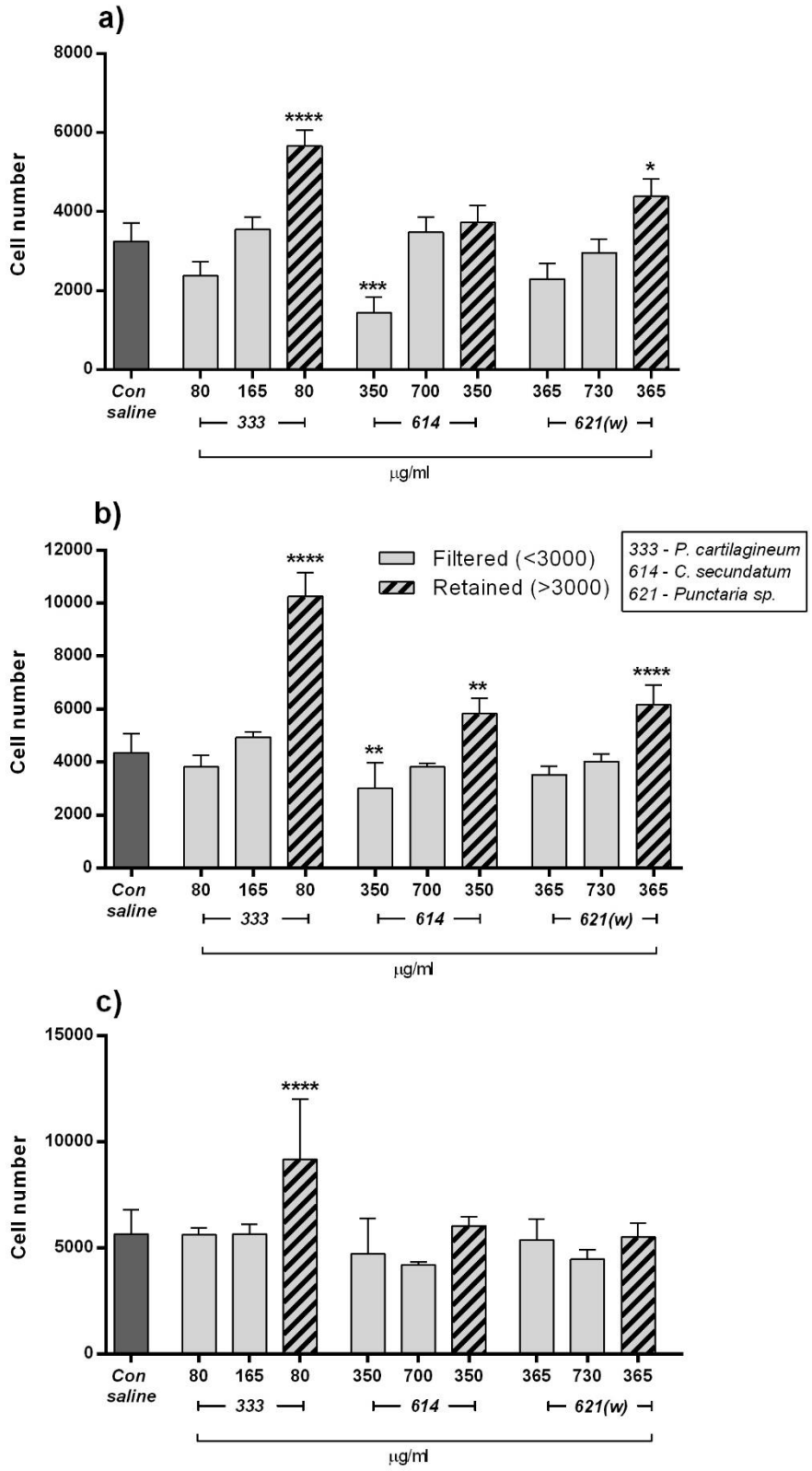


Figure 4.20: hBMSC (donor:005, passage:4) proliferation at day 1 [a)], 4 [b)] and 7 [c)] using crystal violet assay. Control treatment was complete media with saline solution 0.1-10% (Con saline). Cells were also challenged with extracts processed first by ultrafiltration, for extracts 333, 614 and 621. Cell number is presented as mean +/- SD, (n=4). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

4.4 Discussion

This chapter's purpose was to give a better indication of the osteogenic potential of powder extracts, using hBMSCs. Specifically, this focused on measures of cell proliferation, differentiation and mineralisation. It was hoped that this would give a better description of extract activity, allowing those with the best osteogenic and preclinical potential to be selected for *in vivo* testing.

4.4.1 Extraction method

As mentioned in the results section, optimisation of the powder extraction method meant that undissolved extract material was no longer stained by crystal violet in this chapter – eliminating false positive results as an issue. The current works use of an alkaline extraction was briefly justified in the chapter 2 (section 2.4.2). To give more detail, alkaline extractions are not common in similar bioactive research, whilst using it on material which has already been exposed to organic solvents (i.e. original DCM/methanol extraction) makes this studies method even more unique. However, use of conventional solvents such as DMSO and ethanol had already shown limited success in detecting bioactivity (as did use of ethanol to dissolve powders during 'in-house' extractions of extracts 614 and 334). Therefore, trying a new extraction method was a logical progression of the search for osteogenic activity. Furthermore, despite their limited use within the literature, alkaline solutions are generally accepted as a feasible method for extractions from plant sources. Extraction conditions employed in this thesis are based on those used to yield proteins from green tea (Shen et al. 2008). This research found alkaline solutions to be more effective than enzyme based methods, whilst also examining the impact of other variables, including: molarity, temperature, extraction time and weight to volume ratio. 0.1M NaOH solutions at temperatures of roughly 40°C over a period of 2-4 hours were most effective (Shen et al. 2008), all of which were adopted in the current experiment. Such conditions yielded extracts containing 56.4% of green tea proteins, whilst studies on seaweeds also found similar conditions to be optimal for alkaline extractions of intact proteins (P. A. Harnedy & FitzGerald 2013a). In addition to proteins, polysaccharides can also be obtained from alkaline extractions - particularly those required for gels – and often using similar methods (Jiao et al. 2009).

4.4.2 Screening for effects on proliferation

Plocamium lyngbyanum (333), a species of epiphytic red algae, is a good example of an extract treatment which promotes cell proliferation, whilst having no apparent toxic effect. Only three low concentrations of *P. lyngbyanum* were tested for their proliferative effect, including 8, 40 and 80 µg/ml. This decision was due in part to the large number of extracts being tested, necessitating a reduced number of concentrations per extract to ensure experimental design was feasible. Furthermore, initial screening work was conducted partially for this reason – to identify concentrations that had the most effect on cell activity, allowing for focused experimental design. Overall, *P. lyngbyanum* extracts promotion of hBMSC proliferation was notable for its size and repeatability between time points. Concentrations as low as 8 µg/ml were enough to cause a two-fold (or greater) increase in cell number, whilst increasing extract concentration only had a limited further impact on growth. This suggests the presence of a potent bioactive(s), able to stimulate (and potentially saturate) cell proliferation over a period of 7 days. Similar crystal violet results were presented by Kim *et al.* (2015), whereby 0.1-10 µg/ml fucoidan significantly increased hBMSC proliferation over a 3-day time-course. Although Rhodophyta do not contain fucoidan, *P. lyngbyanum* appears to contain a bioactive which produces similar effects. Similarly, Park *et al.* (2016) found that a total algal extract of *Scytosiphon lomentaria* (Lyngbye) Link stimulated proliferation, but to a much lesser extent than that of *P. lyngbyanum*. Furthermore, the concentration required to promote cell growth was much greater than that used in the present study, again supporting the potency of *P. lyngbyanum* treatment.

Ceramium secundatum (614) is another species of epiphytic red algae able to increase cell growth, though its proliferative effect was weaker than that of *P. lyngbyanum*, requiring a 10x greater concentration of 70 µg/ml to promote an increase in cell number. Additionally, a large increase in concentration, to 350 µg/ml, was required to further promote growth. One potential explanation is that *C. secundatum* contains a different active component(s) than that of *P. lyngbyanum*, one which stimulates cell proliferation to a lesser degree. Alternatively, the same active may be present at a lower concentration, or other anti-proliferative molecules could be having an effect. No direct comparisons between this study's treatments and those of other work are possible, as there have been no previous reports about the osteogenic capacity of extracts from either *P. lyngbyanum* or *C. secundatum*. Despite this, the characteristic of increasing proliferation for either extract would be beneficial for an osteoporosis or fracture treatment, as increased osteoblast cell number could potentially stimulate bone formation.

Extracts from *Boergeseniella fruticulosa* (294), *C. spongiosus* (632), *Punctaria sp.* [621(w)] and *Osmundea sp.* (625) were also included in the initial proliferative testing of powder extracts. 621(w), also caused pronounced increases in cell number, at 365 and 730 µg/ml concentrations. Extract solution was available from two water extractions of the powder residue and therefore both were included (two saline extractions were also included for 625), to investigate variability in activity between separate extractions. As shown, treatments from both caused an increase in cell number, though extraction (2) had a greater effect. Variability between extractions was limited as much as possible, however, some degree was still likely, perhaps through slight differences in the timings of particular steps - such as vortexing. Furthermore, whilst the powder to solvent ratio was always 30 mg/ml, the overall amount of powder extract used changed, based on the amount of solution required for each experiment. Within the small area of a 15 ml falcon tube this could have potentially altered powder solubility. Such variability is often reflected in the µg/ml values of table 4.2, which show some substantial differences in the amount of dissolved material between separate extractions [though 621(w) extracts repeatability is relatively good]. Use of large stock solutions was considered to eliminate separate extractions as a source of error, but at this stage of work more experiments (especially *in vivo* work) were still being planned, which may have required use of another solvent. Furthermore, using small extract amounts each time meant that any potential mistakes (such as issues neutralising alkaline solutions or accidental heating above 37°C, which occurred once due to the constant vibration of a low frequency sonicator) would only impact a small portion of available powder residue material.

Another source of potential variability for all hBMSC activity measured within this chapter is interpatient variability. Human-derived derived stem cells are known to show differences in activity between patients, including significant variation in proliferative capacity (Alraies et al. 2017), alkaline phosphatase activity (Chavassieux et al. 1990) and expression of many other markers of differentiation (Verrier et al. 2010). This is likely the cause of variation for day 1 activity of extracts from *C. secundatum* (614) in the first two experiments; as no increase in proliferation was seen in experiment 1, whilst both concentrations caused significant increases in experiment 2. Whilst interpatient variability cannot be eliminated as a variable, ideal experimental design would have accounted for it, through repeat experiments using 3-6 different sets of donor cells. However, this was unfeasible in this study for a multitude of reasons: 1. large repeat experiments (due to extract number) would have been very time consuming, 2. growth of hBMSCs is slower than hFOBs, further limiting/slowing experimental work, and 3. quantity of powders available would not have

allowed a full range of repeat experiments for all extracts (especially mineralisation testing which required much more material).

B. fruticulosa extract (294), had opposing effects at low and high concentrations, significantly stimulating growth at 10 µg/ml and decreasing it at 470 µg/ml. This may indicate the presence of competing bioactives, such as previously suggested for MR-32 (chapter 3). For example, this extract may contain a bioactive which stimulates proliferation, but which is suppressed or outcompeted by another component when at a higher concentration. *Osmundea sp.* extract (625) had no effect on cell proliferation at any concentration. Processing 625 with 0.1M NaOH (and to a lesser extent water) produced an extract that was highly viscous and frothy, especially during mixing. Red algae are known to contain high levels of polysaccharides (Cian et al. 2015), whilst their presence would also explain the viscosity of test solutions. However, as supported by the current study these polysaccharides are unlikely to be bioactive, as Rhodophyta such as *Osmundea sp.* tend to contain cell wall sulphated polysaccharides – such as carrageeenan's and agar – which have shown no previous reports of osteogenic potential (Wijesekara et al. 2011). Finally, *C. spongiosus* (632) extracts sustained decrease in cell number may indicate that it is having a toxic effect. Unfortunately, LDH cytotoxicity assay could not be used to test powder extract toxicity, as solutions contained very fine dissolved material (making them appear slightly cloudy). Whilst this material didn't interfere with CV assay, it did cause false positive LDH readings for some extracts and thus these results have only been included in the appendix (appendix 7). Therefore, there is no way to determine for certain the toxicity of extracts, though CV determined reductions in proliferation give some indication of toxicity; including *C. spongiosus* extract (632), *Ceramium pallidum* [615(w)] at 1520 µg/ml concentration, the 'in-house' extraction of UK 614 extract at 1% and *Sargassum muticum* (840) extract.

Other extracts tested either had a limited effect on proliferation, like 625, or caused decreases in cell number – often at higher tested concentrations. For example, extract 1358, from the deep-sea sponge *Psolus squamatus*, had no proliferative effect at lower concentrations and decreased cell number at 510 µg/ml. Similarly, extract 615, processed from the species *Ceramium pallidum*, was specifically requested after initial powder testing, as red algae – including the related species *Ceramium secundatum* – had stimulated hBMSC proliferation. Unlike *C. secundatum* or *P. lyngbyanum*, this extract did not promote proliferation, though it did have interesting differentiation and mineralisation effects. Likewise, other requested powders, including extracts from the seaweeds *Porphyra linearis* (793) and *Percursaria percursa* (698), as well as a coral from the genus *Lophelia* (137), had no

sustained effect on hBMSC cell number. This indicates several possibilities: 1. no bioactives able to impact cell proliferation were present, 2. bioactives were present but not at a biologically relevant concentration or 3. multiple bioactives had competing effects which negated each other. Regardless, these extracts were screened for differentiation effects before a final subset was chosen for *in vivo* work.

4.4.3 Screening for effects on differentiation

As discussed in chapter 3, osteoblasts undergo a sequential development passing through an early proliferative stage before starting to differentiate to the mature phenotype (Aubin 2001). As ALP is a marker of early differentiation (Miron & Zhang 2012), extract treatments which promote cell maturation would be expected to cause increased ALP activity. Across sets 1 and 2 hBMSCs displayed ALP expression in all treatments tested, including plain media without osteogenic supplementation. For extract set 1 supplementation increased ALP expression at day 7, but not at day 14; whereas during optimisation (chapter 2) addition of supplements to 10% saline solution markedly increased hBMSC ALP activity at days 7, 14 and 21. Similarly, extract set 2 showed no stimulation of activity after supplementation. These differences are most likely due to the aforementioned issue of interpatient variability between the different donor cells. Despite this variation, both hBMSC screens were able to detect extract dependent stimulation or repression of ALP activity. Two screens were performed to give an indication of repeatability, and overall results between the two are very similar.

A good example of an extract which shows a clear differentiation effect is that from *C. secundatum* (614), which was effective at promoting ALP activity at 70 µg/ml, but reduced activity at 350 µg/ml; a reversal of the proliferative trends described. This can be attributed to sequential osteoblast development; whereby proliferative capacity is reduced during differentiation towards the mature phenotype (Aubin 2001). This would explain why 70 µg/ml *C. secundatum* has a limited proliferative effect, but causes the greatest promotion of differentiation. Sequential osteoblast development would also explain why 333, *P. lyngbyanum,* had a limited differentiation effect, as cells were being maintained in a proliferative phase for at least 7 days. This idea is also supported by similar work, such as one study which investigated the osteogenic effect of floridoside [α-D-galactopyranosyl-(1-2)-L-glycerol], derived from the red algae *Laurencia undulata* Yamada (Ryu et al. 2015). This compound exhibited no significant effect on cell proliferation, but strongly promoted cell

differentiation and eventual mineralisation. A range of other extracts have similar effects, such as myricetin (a flavonoid present in fruits and vegetables) (Hsu et al. 2007) and abalone gastro-intestinal extracts (Nguyen et al. 2014). Both extracts had no impact on cell proliferation or viability, but caused dose dependent increases in ALP activity. Taking myricetin as an example, this compound elicited its effect through increased activation of SMAD 1/5/8 and p38 MAPK signalling pathways. However, it should be noted that extracts which promote cell growth can still enhance differentiation, as with fucoidan treatment (Kim et al. 2015). Additionally, nacre WSM is known to cause dose dependent increases in both proliferation (interpreted from protein concentration) and ALP expression of rat bone marrow stromal cells (Pereira Mouriès et al. 2002).

Punctaria sp. water extract [621(w)], which was also previously identified as having a strong proliferative effect, had a similar activity trend to *C. secundatum* extract. For example, low concentration treatment of 73 µg/ml increased differentiation – whereas 365 µg/ml resulted in small decreases of ALP activity but large cell number increases. 73 µg/ml treatment was included in differentiation screening but not proliferation assays (which tested 365 and 730 µg/ml concentrations). This was because 621(w) appeared to have a dose-dependent effect on proliferation, and therefore a lower treatment concentration was predicted to have better differentiation potential (based on the idea of sequential development).

Higher concentrations of extracts from *S. muticum* (840) and *C. spongiosus* (632) inhibit growth and differentiation, perhaps due to toxicity, whilst 6 and 10 µg/ml treatments reduce proliferation but promote cell differentiation. 615 (*C. pallidum*) in saline solution also shows higher ALP activity at 153 µg/ml compared to 765 µg/ml. This could indicate a slight toxic effect at higher concentration, but is hard to determine as cells for the 765 µg/ml treatment detached and were washed away before day 14. This could have been due to toxicity, though seems unlikely as 840 and 632 still displayed ALP activity despite their probable toxic effect. Alternatively, 615 in water was more variable, increasing ALP activity most at 153 µg/ml in extract set 1, layout 1, whilst 761 µg/ml was most active in extract set 2. These differences are most likely caused by variation in levels of active components between the separate extractions of 615(w) used for each experiment (variation between extracts was limited as much as possible, but impossible to completely control for).

The most interesting results of this differentiation screen were for extracts 294, from the red algae *B. fruticulosa*, and 1358 – derived from *P. squamatus*, a deep-sea sponge. Treatments from both these extracts caused pronounced and significant ALP activity increases, but

coupled this with very little proliferative effect. This indicates the presence of a bioactive which stimulates cell maturation, whilst also maintaining growth - or potentially two separate actives. It also reinforces the importance of using multiple measures of activity when determining an extracts bioactive potential.

Extracts from *Porphyra umbilicalis* (792) and *Porphyra linearis* (793) were used interchangeably. This was necessary, as 792 extract solution was too viscous for inclusion during proliferation testing – requiring a lower extract to solvent ratio (10 mg/ml opposed to 30 mg/ml) to mitigate this issue. 793 would have been included in differentiation testing along with 792, but not enough material remained for testing. This, along with both extracts limited effects, meant they were not chosen for further investigation. It was also decided that *Lophelia sp.* (137) extract would not be tested further, due to its lack of effect on hBMSC growth and differentiation. Alternatively, *Percursaria percursa* (698) extract was promising, stimulating cell differentiation in a similar way to *B. fruticulosa* (294) and *P. squamatus* (1358) extracts. However, not enough extract material remained for further testing, with very limited extract stocks at QUB and no remaining material at the Marine Institute. As such, despite its promise, this extract could not be tested in mineralisation or *in vivo* assays.

4.4.4 Screening for effects on mineralisation

Mature osteoblasts are best characterized by their ability to mineralise an extracellular collagen matrix, through production of hydroxyapatite, leading to the formation of new bone (Hadjidakis & Androulakis 2006). As this project focused on identifying a novel osteogenic treatment option it was important to establish the mineralisation potential of each extract, through AR-S staining. Mineralisation work necessitates the use of longer time points and 24 well (rather than 96 well) plates, which require a larger number of cells and a greater volume of extracts to produce treatment solutions. As such, it was necessary focus experimental design (due to limited hBMSC stocks), through use of either day 14 or 21 time points and exclusion of extracts 625, 615 (saline solution excluded, water tested), 137, 792/793 and 698. Excluded extracts were chosen based on those that had shown least potential in proliferation/differentiation screening or those with limited stocks, as previously discussed. Therefore, extracts tested in for mineralogenic effects were: red algae extracts 333, 614, 294, 615(w), 'in-house' extracts of 614 and 334; brown algae extracts 840, 632, 621(w); and an extract from the deep-sea sponge *P. squamatus*.

C. secundatum (614) caused a small non-significant increase in the degree of hBMSC mineralisation, whilst *P. lyngbyanum* (333) caused a significant promotion shown by both visualisation of the dark red staining and subsequent quantification. Considering mature osteoblasts produce factors which control mineral production (Matsuo & Irie 2008), treatments which had the greatest differentiation effect would also be expected to promote mineralisation level the most. Despite this, *C. secundatum* extract coupled its strong promotion of differentiation with a limited mineralisation effect, whilst the opposite was true of *P. lyngbyanum* extract. This could again be due to differences in extraction solution used between experiments, though even with this variability a greater mineralisation level would be expected for *C. secundatum* extract. Another potential explanation is that *P. lyngbyanum* extract treatments mineralisation potential is due to promotion of cell number. This growth promotion would allow 100% confluency to be reached quickly (as cells were first treated with extract solutions when they reached 75% confluency), limiting further growth. Cell-cell contact in confluent monolayers creates contact inhibition, stabilising monolayers (Pautke et al. 2004), and is known to aid cell maturation, due to the effect of osteogenic factors which require contact between cells (Yamada et al. 2016). This is similar to the idea of macromolecular crowding, whereby macromolecules mimicking crowded *in vivo* conditions are known to cause increased ECM deposition by human corneal (Kumar et al. 2018) and dermal (Satyam et al. 2016) fibroblasts.

Another inconsistency is that for both *C. secundatum* and *P. lyngbyanum* extracts the highest treatment concentration gave the greatest mineralisation level increase, whereas cell differentiation peaked for both extracts at lower concentrations. Relevant studies show examples where increases in ALP and mineralisation level are tightly correlated, as with fucoidan (Y.-S. Cho et al. 2009; Hwang et al. 2016). However, as previously mentioned, there are also examples of other trends, such as with floridoside (Ryu et al. 2015). This extract displayed a similar trend to that of the present study, whereby the highest floridoside concentrations promoted the greatest degree of mineralisation in murine bone marrow mesenchymal cells, but not always greatest ALP activity. It therefore appears that, like with floridoside, both extracts have their greatest effects on mineralisation and differentiation at different concentrations, perhaps reflecting extract composition. For example, as extracts are a crude mixture, mineralisation may be promoted by a different bioactive to that which supports hBMSC differentiation – which is more active at a higher concentration.

Remaining extracts tested for their mineralisation potential can be split into two groups; those that notably increased hBMSC mineralisation and those which did not. Of these, the

first group is the largest and comprises extracts the red algae extracts *C. pallidum* [615(w)] and *B. fruticulosa* (294), brown algae extract *Punctaria sp.* [621(w)], and the deep-sea sponge *P. squamatus* (1358) extract. These extracts are similar in that their maximum concentrations caused the greatest increase in mineralisation level, though variations in activity do exist between them. For example, 615(w) had no effect at the day 14 time point, whilst some extracts also increased mineralisation at concentrations other than the maximum tested. Furthermore, this group of extracts had similar effects on other measures of cell activity – promoting differentiation but having little effect on proliferation. *Punctaria sp.* is the only exception to this, as it also significantly promoted cell growth. Overall, these extracts join those already described in the literature which are able to promote *in vitro* mineralisation; such as molluscan extracts like nacre (Brion et al. 2015) and other shell derived proteins (Hyung et al. 2016), previously discussed algal bioactives (Kim et al. 2015; Ryu et al. 2015) and other marine derivatives like Aquamin (Gorman et al. 2012) and abalone gastro-intestinal extracts (Nguyen et al. 2014). Such effects are particularly promising whilst searching for a bioactive to aid bone healing, as they may provide a treatment option to stimulate bone formation in osteoporotic patients or those suffering from complex fractures or delayed/non-union.

The second group, those treatments which did not stimulate *in vitro* mineralisation, include extracts from the brown algae *S. muticum* (840) and *C. spongiosus* (632). Both extracts also failed to stimulate proliferation and only promoted differentiation at their lowest concentrations. This is most likely caused by a suppressive effect, which appears to limit cell development. Toxicity is also a possibility, but is unlikely considering that a limited number of non-mineralising cells survived 21 days of exposure to both extracts. Despite their limited *in vitro* effects, these extracts [along with Osmundea sp. (625)] will also be taken forward to *in vivo* testing as "negative controls" - in order to test the ability of *in vitro* tests to predict *in vivo* activity.

4.4.5 Effects of 'in-house' extracts

To test reproducibility, fresh sample material was also sourced for the active extracts *C. secundatum* (614) and *Plocamium cartilagineum* (334). In-house extractions were undertaken specifically to show that bioactivity could be maintained between separate extractions of sample material. Material was collected from two sample locations, Donegal on the north-west coast of Ireland and Dorset on the south coast of England (approximately

500 miles apart). This allowed determination of potential differences in activity based on sample location, whilst both were collected within 3 days of each other to eliminate seasonality as a variable. Unfortunately, no species information was available for extract 621, despite its promising activity, and therefore fresh material could not be sourced. Extra powder material, solvent dissolved material, original samples and voucher specimens were all requested from the Marine Institute with the hope of determining 621 species information, but no material remained in any form. Existing powder extract material for 621 was sent for DNA identification, though amplification of the COX1 gene was unsuccessful and results for more specific brown algae primers are still pending. Furthermore, *Plocamium cartilagineum* (334) was collected rather than the originally used *Plocamium lyngbyanum* (333), as no *P. lyngbyanum* was available at either sample site. Though not ideal, both species are very similar, with *P. lyngbyanum* a recently described species which was previously accepted as being a molecular entity equivalent to *P. cartilagineum* (Cremades et al. 2011). Differences between the two are based mainly on morphological aspects such as thallus colour and ramuli per series, as well as some habitat variation, and therefore molecular composition between them is likely very similar.

UK-derived material of extract 334, from *P. cartilagineum*, had a similar activity profile to original 333 extract (*P. lyngbyanum*), with all concentrations increasing hBMSC cell number; whilst only the highest concentration of Irish-derived material had a stimulative effect. Overall, this shows that both extracts 333 and 334 contain compounds/molecules which have a similar proliferative effect. Irish derived powder extract showed more variation compared to UK and original extract material, particularly at lower concentrations which were less active. These differences are unlikely to be due to species variation of active components, as UK derived *P. cartilagineum* (334) extract performed similarly to *P. lyngbyanum* (333). Likewise, seasonality is not likely to be an issue, as UK and Irish-derived samples were collected between 10-13th September 2016 – whilst original 333 sample material was collected on August 27th 2011. More likely, was that less of the active component(s) was present, potentially due to differences in extraction efficiency or habitat based variation in algal compound/molecule composition. This would explain why a higher concentration of 117 µg/ml (compared to 9 µg/ml for UK sample) was required to significantly promote cell number.

Ethanol was also used to create both solvent dissolved and powder extracts for 334 and 614. This was performed to test if one extraction solvent, as opposed to two (DCM and methanol), had an impact on activity levels; potentially leaving more material behind as a powder

residue. Ethanol was chosen as it had been used previously (chapter 3), showing low toxicity with hBMSCs. Compared to 'powder extract normal' – extracted with DCM/methanol – the activity trend was reversed, with no UK effect but a dose-dependent increase in activity for Irish-derived powder extract. As such, this extract appears to contain a bioactive which stimulates proliferation, which is likely different from that present in 'powder extract normal' - as this material promoted cell number with both UK and Irish-derived extracts.

As with the two species of *Plocamium*, 'in-house' *C. secundatum* (614) extract was tested at an equivalent concentration range to that used with original 614. However, cell proliferation with 'in-house' 614 was much different to that previously described, with no sustained increases in cell number at either tested concentration for both UK and Irish-derived material. Furthermore, the obvious reduction with 525 µg/ml (UK) treatment at day 4 indicates the presence of a compound/molecule able to suppress growth at a high concentration. Overall, proliferative bioactives present in the original extracts may not have been extracted, could be present at a much lower concentration or their activity may be being masked by another suppressive molecule. Whilst disappointing, this loss of activity is less surprising than it would have been for 334, as original 614 extract had less of a proliferative effect; which would have been more easily lost/masked with subsequent extractions. Reasons for this loss of activity are numerous, though are most likely due to variation in algal extract composition – caused by different conditions between sample locations (i.e. temperature and intertidal position) – or seasonality, as original 614 samples were collected on June 5th; compared to September 10-13th for 'in-house' extracts. An excellent example of this variability is a study by Rødde *et al.* (2004), which investigated effects of both seasonality and geographical location on chemical composition of the red alga *Palmaria palmata* (L.) Kuntze. It was shown that composition of major constituents like proteins and low molecular weight carbohydrate varied with season, including between early and late summer. Furthermore, all major constituents had variation based on geographical location, which (amongst others) included sample sites in Northern Ireland (Portaferry) and Ireland (Galway). In addition to this study, seasonal and geographic variation in the composition and activity of algal extracts is well documented. For example, macroalgae extracts from a variety of species are known to show variation in antifouling (Hellio et al. 2004; Maréchal et al. 2004), antibacterial and antifungal activity (Stirk et al. 2007), based on the season they were collected in. Similarly, geographic variation, both small and large scale, is known to affect the levels of secondary metabolites like phenolic compounds (Van Alstyne et al. 1999) and halogenated furanones (Wright et al. 2000), most likely due to variation in

nutrient and light levels. Returning to results of the present study, 614 'powder extract ethanol' was also disappointing, reducing cell number to varying degrees at all tested concentrations – indicating a suppressive effect of extracted compounds/molecules. Overall, despite 334 (Irish) showing good activity in ethanol, this solvent was not effective enough to justify its use with all extracts.

For 'in-house' extractions both DCM/methanol and ethanol dissolved fractions were also tested for proliferative activity, after reconstitution in ethanol. Compared to powder extracts these treatments had very little effect on cell number. 0.5% DCM/methanol extract of 614 (Irish) significantly increased cell number, though this is almost certainly an anomaly caused by the large variability across repeats. Alternatively, 0.5% ethanol extract of 614 (Irish) contained components able to significantly reduce hBMSC cell number. Other than these exceptions solvent dissolved material caused little variation in activity, supporting the results of chapter 3 and the decision to focus on powder extracts instead.

Finally, 'In-house' extracts of *C. secundatum* (614) and *P. cartilagineum* (334) were particularly notable in their mineralisation effects, causing very large promotions in mineralisation level at all tested concentrations. For *P. cartilagineum* (Irish, 117 µg/ml) this resulted in a complete covering of the cell monolayer with stained calcium deposits, making individual nodules hard to distinguish. This effect correlates with that of *P. lyngbyanum* (333), though the higher overall mineralisation level may indicate that more bioactive material is present – or potentially the presence of a more potent molecule/compound. *C. secundatum* 'in-house' extract greatly exceeds the mineralisation effect seen with original extract material, indicating more of the original active, or a completely new one, was extracted from these samples. This is not unexpected, considering the aforementioned differences in proliferation effects between original and 'in-house' extract material, as well as sampling and extraction method variables. However, it is important to note that Irish and UK-derived 'in-house' extracts had very similar effects, indicating a similar composition despite their vastly different sample sites. It may be that use of fresh extract material, as opposed to that stored since June 2012 and exposed to repeat freeze/thaw cycles, may have better preserved bioactive structure, explaining the activity seen with 'in-house' compared to original extract material. Unfortunately, ALP activity assay results are not available for comparison to both sets of 'in-house' extracts. However, it can be hypothesised that extracts from both species would likely have caused large increases in ALP activity levels, as such pronounced effects on mineralisation would likely not have occurred without significant early cell differentiation.

4.4.6 Subfraction testing

Of those extracts tested, those from *C. secundatum* (614), *P. lyngbyanum* (333) and *Puncatria sp.* [621(w)] were most promising, solely in terms of their stimulative effect on cell number. As such, these extracts were also processed via ultrafiltration, to give an indication of the size of their bioactive components. This produced two separate solutions – one containing material which passed through the filter (<3000 NMWL portion) and one comprised of retained material (>3000 NMWL portion). More extract material passed through the filter than was retained and thus only one retained concentration was able to be tested, compared to two for filtered material. Overall, filtered extract treatments had no effect on cell number, whereas retained treatments performed better - particularly at day 4 whereby all three extracts significantly increased growth. This was especially true for 333, indicating that its bioactive component(s) is greater than 3000 NMWL in size – such as DNA > 25 base pairs (BP) in length or proteins > 15k molecular weight (MW). However, though increased at day 4, extracts 614 and 621(w) showed much less activity than in previous tests. One potential reason for this is damage to compound structure due to shear during ultrafiltration, which may have altered compound activity. Nevertheless, this is more often reported in large scale industrial processing or with long centrifugation times (Thomas & Geer 2010), and therefore seems an unlikely issue in this work (0.5 ml solution centrifuged for 5 minutes). Another technical limitation may have been the unfolding of structures (i.e. proteins) and their aggregation at the membrane interface (Cromwell et al. 2006). More likely is that the concentration of retained test solutions were too low to reach previous activity levels, or had only displayed significant activity as a mixture. Of all potential explanations a mixture seems most likely, as both 614 and 621(w) had displayed significant activity before processing.

4.5 Summary

A succinct summary of testing on so many extracts is difficult, as each presented different trends for each measure of *in vitro* activity. This chapter detailed powder extract potential in terms of hBMSC proliferation, differentiation and mineralisation. For the most part these extracts had first shown potential during testing with hFOBs, and therefore a small range of promising concentrations was selected for use with hBMSCs. Most of those extracts tested displayed significant positive effects on at least one or more measures of cell activity. For example, *P. lyngbyanum* caused significant and notable promotions of proliferation, differentiation and mineralisation at different tested concentrations, indicating its excellent pre-clinical potential. Even extracts such as *S. muticum* and *C. spongiosus*, which had no

proliferative or mineralogenic effects, still significantly promoted cell differentiation at their lowest concentrations. As such, the majority of extracts tested within this work will be included within future testing. Exceptions to this include seaweed extracts *P. umbilicalis* (792) and *P. linearis* (793), as well as the coral *Lophelia sp.* (137), which were excluded from future testing due to their limited effects on hBMSC activity. Furthermore, despite its ability to promote ALP activity levels, limited material was available for *P. percursa* (698) extract and therefore it will not be included in future work. Those extracts which showed potential and will be tested further include: the red algae *C. secundatum* (614), *C. pallidum* (615) *P. lyngbyanum* (333), *P. cartilagineum* (334) and *B. fruticulosa* (294); the brown algae *Punctaria sp.*; and the deep-sea sponge *P. squamatus* (1358). Those extracts which didn't show great potential, but will be included as negative controls, include: the brown algae *C. spongiosus* (632) and *S. muticum* (840) and the red algae *Osmundea sp.* (625).

Overall, powder extracts tested within this chapter show highly promising *in vitro* potential, despite being the material left over from original organic solvent extractions. Experimental work within the remainder of this thesis will now describe the *in vivo* potential of each extract, specifically their ability to promote the growth bone and regeneration of zebrafish bone. This will indicate if extract effects are maintained within a whole organism and will also give a better indication of their pre-clinical potential.

Chapter 5

In vivo bone growth and regeneration

5.1 Introduction

In vitro methods are generally used to screen for bioactive potential, before deciding on further testing – of which *in vivo* studies are a key component. Such testing allows confirmation that activity – like osteogenic potential – is maintained within a whole organism. Furthermore, it allows the effect of physiological conditions on an extract/compound to be determined and gives an indication of tissue response to a particular bioactive (Pearce et al. 2007).

Traditionally, *in vivo* models within this field focus on mammals, such as ovariectomised mice, rabbits and sheep - which are commonly used for assessing the osseointegration of implants (Alghamdi, van den Beucken & J. a. Jansen 2014), though they are also used in studies testing extracts. It should be noted though that mammalian models are generally very different to each other and therefore difficult to compare (Clarke & Jordan 2008). One extract based study tested mycoepoxydiene, a compound isolated from a marine fungus (Diaporthe sp. HLY-1), showing it to reduce ovariectomy-induced bone loss (Zhu et al. 2013); a model which is useful for determining the activity of potential systemic treatments for osteoporosis. Furthermore, after well characterised *in vitro* testing, water soluble nacerous factors (from *Pteria martensii*) were shown to help prevent osteoporotic bone loss through osteoclast inactivation in mice (Kim et al. 2012). Of particular relevance to this work, algal extracts have also been tested *in vivo* – such as that of *Sargassum horneri.* This was shown to cause a significant increase in bone components of femoral diaphyseal and metaphyseal tissues in both young and aged rats (Uchiyama & Yamaguchi 2002). Similar work demonstrated the potential of other marine algae to increase bone calcium content of mice, including *Undaria pinnatifida*, *Eisenia bicyclis* and *Cryptonemia scmitziana*, though *S. horneri* had the best overall effect (Yamaguchi et al. 2001). Of note, whilst ovariectomy is common it is not the only type of *in vivo* model used. For example, fracture models can be used, to test bone healing or the performance of synthetic bone substitutes (Pearce et al. 2007), and diet can also be manipulated – such as in one study testing Aquamin. In this work, mineral rich extract of *Lithothamnion calcareum* (Aquamin) was shown to reduce bone defects in mice fed a high fat western diet (Aslam et al. 2010).

Overall, use of mammals such as mice and rabbits give a good indication of *in vivo* skeletal response to a material or extract, as evidenced by their common usage and similarities to human bone (Pearce et al. 2007). However, no model organism, no matter how closely related, is ever a perfect replacement for testing using a human subject. Furthermore,

mammalian models suffer from practical concerns, such as long generation times and low fecundity, strict ethical regulations and high running costs, which can act as a barrier to experimentation. One increasingly popular alternative is zebrafish, *Danio rerio* (Hamilton, 1822), a small freshwater fish which lives in tropical regions of South and Southeast Asia. At first comparison, mammals - being higher vertebrates and more closely related to humans – are obviously preferable *in vivo* models. Despite this, zebrafish have many characteristics that make them suitable for use, particularly for testing multiple different extracts (as in this study). Firstly, despite the last common ancestor between zebrafish and humans existing approximately 420 million years ago, there are many similarities between teleost and mammalian skeletons (Laizé et al. 2014). For example, both skeletons are comprised of many identical bones, formed via a cartilaginous anlage being replaced by bone - through both endochondral and dermal ossification. Furthermore, there is also a good conservation of developmental events and key bone formation regulators between the two (Laizé et al. 2014). Additionally, there are practical benefits to using zebrafish, including: 1. high fecundity and short generation times (meaning many juvenile and adult individuals can be quickly sourced for testing), 2. they are low cost, 3. *in vivo* assays using zebrafish tend to be quick and high throughput, and 4. robustness – individuals are easy to manipulate and quick to adapt to different conditions (Laizé et al. 2014). Based on these characteristics it was feasible to test multiple extracts identified as having osteogenic potential within this work (chapter 4), despite the relatively early stage of their development. Within similar studies there is a general tendency to test 1 active/compound at a time, after detailed chemical and *in vitro* characterisation – most likely due to costs associated with mammalian models. However, this risks activity being lost within an organism and necessitates use of a smaller number of compounds, and was therefore not the approach used in this work.

Overall, zebrafish have been used as a model organism for over 100 years, with early work focusing on embryogenesis (Sullivan & Kim 2008). In the 1970's genetic research began, but it was not until the last 20 years that zebrafish use drastically increased, as its application in testing for different activities became apparent (Langheinrich 2003). This increase in research intensity is partly due to the aforementioned benefits of zebrafish, such as their high fecundity, low cost and ability to be used for rapid screening (Parng et al. 2002). However, they also benefit from having a fully sequenced genome, and are particularly useful for drug discovery and testing due to their permeable and transparent embryos (Kari et al. 2007). Cardiovascular, anti-angiogenic and anti-cancer drugs have all shown comparable responses in zebrafish compared to mammalian systems (Langheinrich 2003; Bakkers 2011), as have

toxicity and tetragenic effects (Parng et al. 2002). Of particular relevance to this work, zebrafish, and to a lesser extent medaka, are also used to test osteogenic and osteotoxic molecules through several separate systems; of which two were used in this phase of work to screen for *in vivo* osteogenicity. Firstly, operculum (gill bone) area changes were assessed in larval zebrafish, to quantify an extracts effect on bone formation (Tarasco et al. 2017). Secondly, regeneration of adult caudal fins was used to determine effects on bone regeneration (Cardeira et al. 2016). Both systems, especially the operculum, are robust, reliable and novel measures of *in vivo* skeletal response to extract treatment, which will be discussed in further detail throughout this chapter.

This chapter describes the *in vivo* osteogenic potential of promising powder extracts, as identified in chapter 4. Specifically, extract effects on bone formation, as assessed through the operculum area changes of larval zebrafish, are determined in detail for a large number of extracts. A smaller subset was also tested for *in vivo* potential using a system of adult caudal fin regeneration.

5.2 Methods

5.2.1 Extracts tested

5.2.1.1 Extracts tested - operculum area in juveniles

Table 5.1 details extracts that were tested using the operculum area system in juvenile zebrafish. Results from each experiment are presented in the order extracts are listed in this table. Sample sizes are reported as those planned at the start of experiments and therefore do not include mortality – mortality figures are presented for each test in the results section.

Table 5.1: list of all extracts tested using the operculum area system in juvenile zebrafish, including the genus and species for each. Also included is information on whether a water or saline extract was tested, along with sample sizes for each experiment.

			Sample size	
Extract	**Genus/species**	**Water or saline extract?**	**Water**	**Saline**
614	*Ceramium secundatum*	Both	15	15
614 'in-house'	*Ceramium secundatum*	Saline		15
615	*Ceramium pallidum*	Both	15	15
333	*Plocamium lyngbyanum*	Both	15	15
334 'in-house'	*Plocamium cartilagineum*	Saline		15
294	*Boergeseniella fruticulosa*	Both	10	15
625	*Osmundea sp.*	Both	15	15
621	*Punctaria sp.*	Water	15	
840	*Sargassum muticum*	Both	10	10
632	*Cladostephus spongiosus*	Both	15	10
1358	*Psolus squamatus*	Both	15	10

5.2.1.2 Extracts tested – caudal fin regeneration in adults

Table 5.2 details extracts that were tested using the caudal fin regeneration system in adult zebrafish.

Table 5.2: list of all extracts tested using the caudal fin regeneration system in adult zebrafish, including the genus and species for each. Also included is information on whether a water or saline extract was tested, extract concentration and sample sizes for each experiment.

Extract	Genus/species	Extraction	Concentration (µg/mg fish weight)	Sample size
1358	*P. squamatus*	Water	0.025	5
632	*C. spongiosus*	Water	0.13	7
632	*C. spongiosus*	Water	0.25	5
614	*C. secundatum*	Water	0.51	6
621	*Punctaria sp.*	Water	0.93	6
632	*C. spongiosus*	Water	0.25	5
632	*C. spongiosus*	Saline	1.3	5
614	*C. secundatum*	Saline	1.12	5
333	*P. lyngbyanum*	Saline	0.82	5
632	*C. spongiosus*	Saline	0.65	5
615	*C. pallidum*	Saline	1.56	5
294	*B. fruticulosa*	Saline	0.68	5

5.2.2 Extract preparation

Water or saline dissolved extracts tested during this chapter were produced using the method detailed in chapter 4. Briefly, powder extracts were dissolved in either water or 0.1 M NaOH at a concentration of 30 mg/ml, before being dissolved (including vortexing, heating, sonication and rotation). Solutions were then centrifuged twice to remove undissolved material and the resulting supernatant was pH tested and neutralised, via addition of weak (0.1-0.5 M) HCl or NaOH. Final extract concentrations were determined using the method detailed in chapter 4 (section 4.2.2.4) and are stated in all figures.

For operculum area studies, larvae were constantly exposed to treatments during the test period; through submersion in 10 ml of water containing dissolved extracts. For each experiment this treatment water was prepared by first adding extract solution at the maximum test concentration, followed by a dilution series to achieve the lower concentrations. Concentrations were chosen based on a small pilot study using *P. squamatus* and *C. spongiosus* extracts (results not shown) and generally mimic those used *in vitro*, though maximum test concentrations were higher than those of cell work.

For caudal regeneration tests, adults were injected with extract solutions rather than being submerged in them. These intraperitoneal injections were limited to a 3 µl volume per 100 mg fish weight, to prevent undue swelling and damage to test individuals. Therefore, extract solutions had to first be concentrated, to ensure that individuals would be treated with a

relevant amount of extract material. To accomplish this, a known volume of each extract solution was transferred to a pre-weighed Eppendorf tube, with 5 holes added to the lid to allow sublimation to occur in a freeze dryer Christ Alpha RVC vacuum centrifuge coupled to a Christ Alpha 1-4 freeze dryer, Germany) for 24 h (settings: -45°C, 10 mbar pressure, 300 g). Drying time was > 4 hours. Afterwards, each Eppendorf was re-weighed to determine dried extract weight. Saline or water solvent was then added to each tube, at volumes calculated so that the maximum injection volume (3 µl/100 mg fish weight) would give an extract concentration which approximated to that used *in vitro*, or during operculum testing.

5.2.3 Husbandry

Water conditions for all test individuals were as follows: pH 7.5 ± 0.1, conductivity 700 ± 50 µS, NH_3 and NO_2 lower than 0.1 mg/L, NO_3 at 5 mg/L. This water was produced by adding sodium bicarbonate and salt mixture (Instant Ocean, Blacksburg, VA) to reverse osmosis treated water. Fish were fed twice daily, once with *Artemia* nauplii (EG strain from INVE Aquaculture, Dendermonde, Belgium) and once with commercial dry food (Zebrafeed from Sparos LDA, Portugal). Photoperiod was set to 14 h light and 10 h dark.

Individuals used in the caudal fin regeneration system were adult AB wild-type strain zebrafish, aged between 3 and 5 months. For operculum testing, sexually mature AB wild-type strain zebrafish were mated, and fertilised eggs were collected. Larvae hatched from these eggs were used in testing.

5.2.4 *In vivo* operculum area system

Operculum area was determined in zebrafish larvae (figure 5.1) by measuring operculum area after a three-day exposure period to extract treatments, following a method described by Tarasco et al. (2017).

Fertilised eggs were collected and transferred to a 1-L container, to which methylene blue (0.0002% w/v) was added to prevent fungal growth. Eggs were then placed in an incubator (28.0 ± 0.1°C) for 72 h with a photoperiod of 14-10 h light-dark.

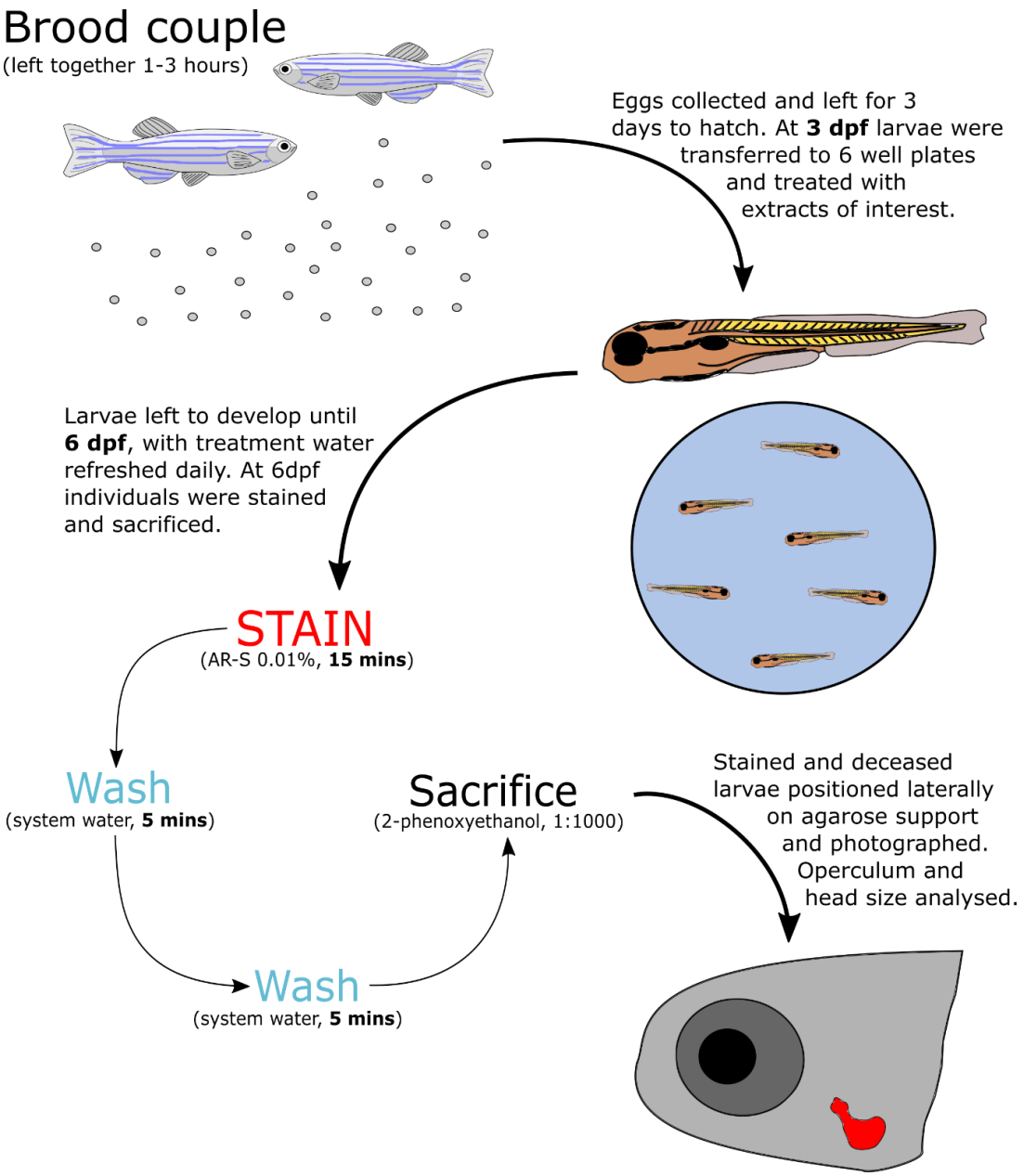


Figure 5.1: illustration depicting the method used to assess the *in vivo* osteogenic potential of extracts in larval zebrafish. Key steps from the whole process are included, from collection of fertilised eggs produced by a brood couple through to image acquisition at 6 days post fertilisation (dpf).

At three days post-fertilisation (dpf), viable larvae were transferred into 6-well plates. During experimentation each well housed 15 larvae in 10 ml of system water. Upon initial exposure, larvae were placed in wells with 3 ml of system water, to which a further 7 ml - containing dissolved extracts - was added. Each experiment had a vehicle control group, which exposed larvae to the maximum concentration of solvent used with extract treatments - either 10%

water or saline solution dissolved in system water. Furthermore, a positive control group was also included in each experiment, consisting of either 10, 15 or 20 pg/ml calcitriol (Tocris, Bioscience) dissolved in ethanol, as well as a negative control for this group – containing only 0.1, 0.15 or 0.2% ethanol. Differing positive control concentrations were necessary as calcitriol degrades in light, meaning its activity decreased after repeated use during the 4-month study period; thereby necessitating higher concentrations. 70% of treatment water was replaced daily with that containing fresh extract treatments, during the three-day exposure period. Subsequently, larvae were stained in excess AR-S solution (0.01%, pH 7.4) for 15 min, washed twice in system water (5 min) and then sacrificed (1:1000 dose of 2-phenoxyethanol dissolved in system water; Sigma-Aldrich, UK).

Larvae were sacrificed in batches of 15 individuals and imaged (via lateral placement on a 2% agarose gel) directly after euthanasia, to limit tissue degradation. An MZ 7.5 stereomicroscope (Leica, Wetzlar, Germany) was used, equipped with a green light filter (λ_{ex} = 530–560 nm and λ_{em} = 580 nm) and a black-and-white F-View II camera (Olympus, Hamburg, Germany). Image parameters were as follows: exposure time 2 s, gamma 1.00, image format 1376 x 1032 pixels, binning 1x1. Images were subsequently analysed using image J 1.49v software. Firstly, the red channel images were isolated before measurements of the head and operculum were taken using built-in tools on image J (see example in figure 5.2). Ratios comparing the head and operculum area were then calculated and given as a percentage of the control. The following two equations were used to calculate the change in operculum size relative to control individuals:

(1) $Ratio = \frac{operculum\ area}{head\ area}$

(2) $operculum\ size\ (\%\ of\ control) = \frac{ratio}{average\ control\ ratio} \times 100$

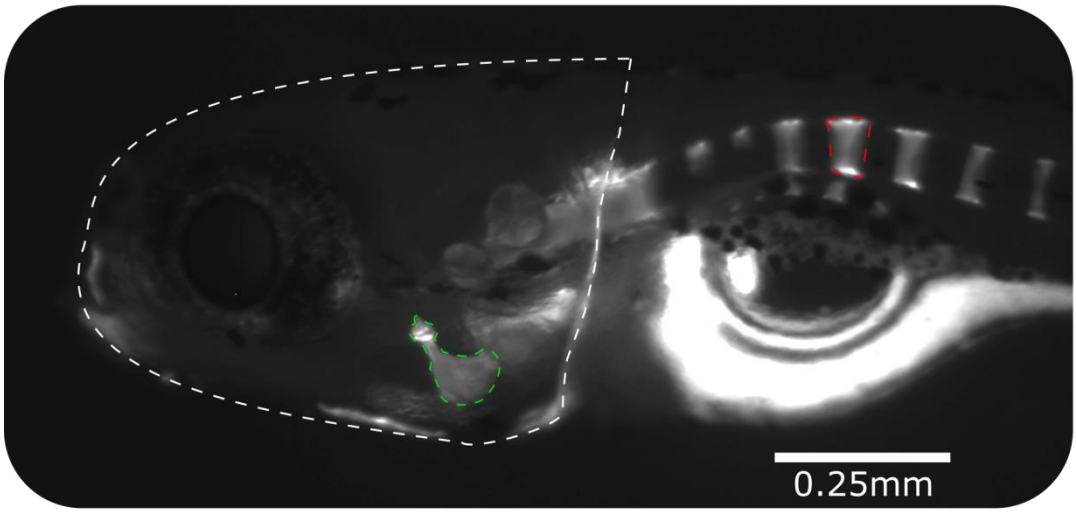


Figure 5.2: example image of a larvae stained with AR-S, for which the red channel image has been isolated. Dotted white lines indicate the head area (as measured in image J), whilst green is the operculum. One vertebral segment has also been highlighted (red dotted line), as whilst not directly measured vertebrae also give a good indication of the mineralisation potential of extracts.

5.2.5 *In vivo* caudal fin regeneration system

Caudal fin regeneration experiments used a method modified from Cardeira et al. (2016). Adult individuals were anesthetised via submersion in a water bath containing MS222 (Sigma-Aldrich, St. Louis, MO) and subsequently quickly dried and weighed. Injection volumes were calculated for individual weight, based on a target addition of 3 µl solution per 100 mg fish weight. For each experiment treatment solutions included concentrated dissolved extracts and a vehicle control – either saline solution or water. After weighing, caudal fin was amputated 1-2 segments anterior to the bifurcation of the most peripheral branching lepidotrichia. After amputation, individuals were inverted (ventral side up) and placed within a foam holder so that treatments could be administered via intraperitoneal injection. Individuals were then placed into plastic containers holding 900 ml of water, at a density of 5-7 fish per container. 50% of container water was renewed daily and heated to 33°C to accelerate the regenerative process (Boominathan & Ferreira 2012). Fish were fed twice daily, once with *Artemia* nauplii (EG strain from INVE Aquaculture, Dendermonde, Belgium) and once with commercial dry food (Zebrafeed from Sparos LDA, Portugal). Photoperiod was set to 14 h light and 10 h dark.

After a five-day period, fish were live-stained by submersion in 200 ml of 0.01% AR-S solution for 15 min, then washed for 5 min before being anesthetized in a solution of MS222 (Bensimon-Brito et al. 2016). Caudal fin areas were then immediately imaged, using the same camera system and settings as described for the larvae (except for exposure time which was

set to 500 ms). Fluorescence and bright field images were collected for each individual. Images were then analysed using Image J 1.49 software, allowing several measurements to be taken. Total regenerated area (REG) was measured from the amputation plane to distal end of the fin and was corrected with the stump width (STU). Mean ray width (RAY) was determined as the mean width of rays at the first intersegment joint below the amputation plane. Real mineral area (RMA) was measured after applying a colour threshold which selected only the mineralised red areas. Finally, the intensity of calcium staining was assessed using the intensity of pixels within mineralised regions (using the YUV colour model and intensity values in the 5 - 254 arbitrary unit range).

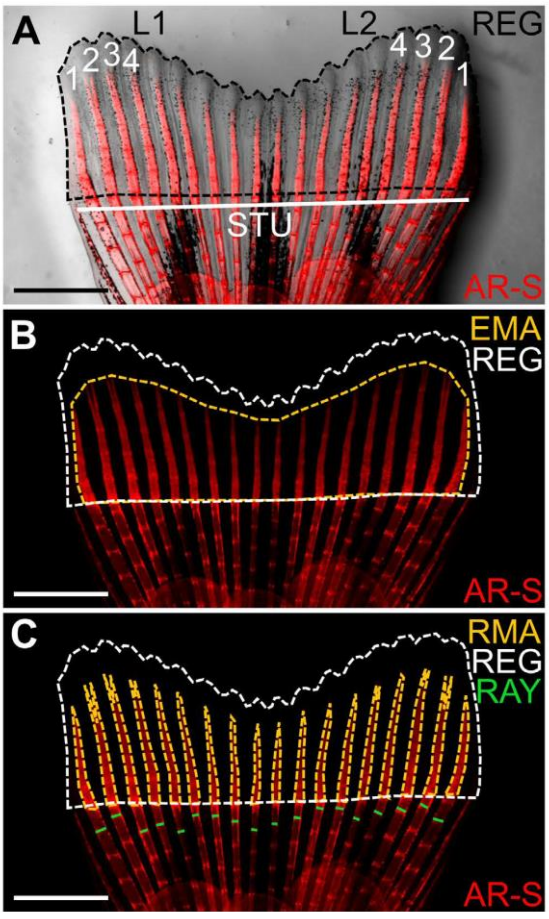


Figure 5.3: from Cardeira et al. (2016). (**A**) Inter-specimen variability of the regenerated area (REG; outlined by the black dashed line) is corrected with the stump width (STU). Numbers (1–4) show the designation of the lepidotrichia from each of the two fin lobes (L1 and L2). (**B**) Estimated mineralized area (EMA; outlined by the yellow dashed line) corresponds to the area comprising the mineralized lepidotrichia and the inter-ray space. (**C**) Real mineralized area (RMA; outlined by the yellow dashed lines) corresponds to the area stained with alizarin red S, excluding the inter-ray space. The measurements necessary to calculate the mean ray width below the amputation plane (RAY) are also presented.

Previously detailed measurements were then used to calculate the degree of osteogenesis and regeneration associated with each treatment and control, using two equations:

$$(1)\ Regeneration = \frac{Total\ regenerated\ area\ (REG)}{Stump\ width\ (STU)}$$

$$(2)\ Osteogenesis\ = \frac{Real\ mineral\ area\ (RMA)}{Mean\ ray\ width\ (RAY)} / \frac{Total\ regenerated\ area\ (REG)}{Stump\ width\ (STU)}$$

In addition to these equations, pixel intensity data was collected for arbitrary unit values between 5 -254, though generally only a limited number of pixels were present above a value of 50. Subsequently, an overall range was chosen for analysis, based on the spread of values seen, which was generally between arbitrary unit values of 7 - 30. Within this range pixel intensity values were converted to percentage values. Subsequently, histograms of average percentage values were then plotted for each treatment group. Finally, two pixel intensity classes were chosen from this range (one low and one high). Intensity classes were again chosen based on the spread of values seen within an experiment, though were generally comparable between experiments. For an individual within a treatment group the mean pixel intensity for each class was then calculated. Values for each test subject within a treatment group were then averaged and presented as 'average percentage frequency' for each class; allowing for comparisons of changes in pixel intensity (as a measure of bone density) between control and extract treatments.

Of note, caudal fin regeneration experiments had to be smaller in scope compared to those using the operculum system, as adult individuals needed more space and were more difficult to initially treat with extracts (fish can become overly stressed if this process takes too long). As such, fewer extracts were tested using this system, whilst lower sample sizes were also used for each treatment group (see table 5.2)

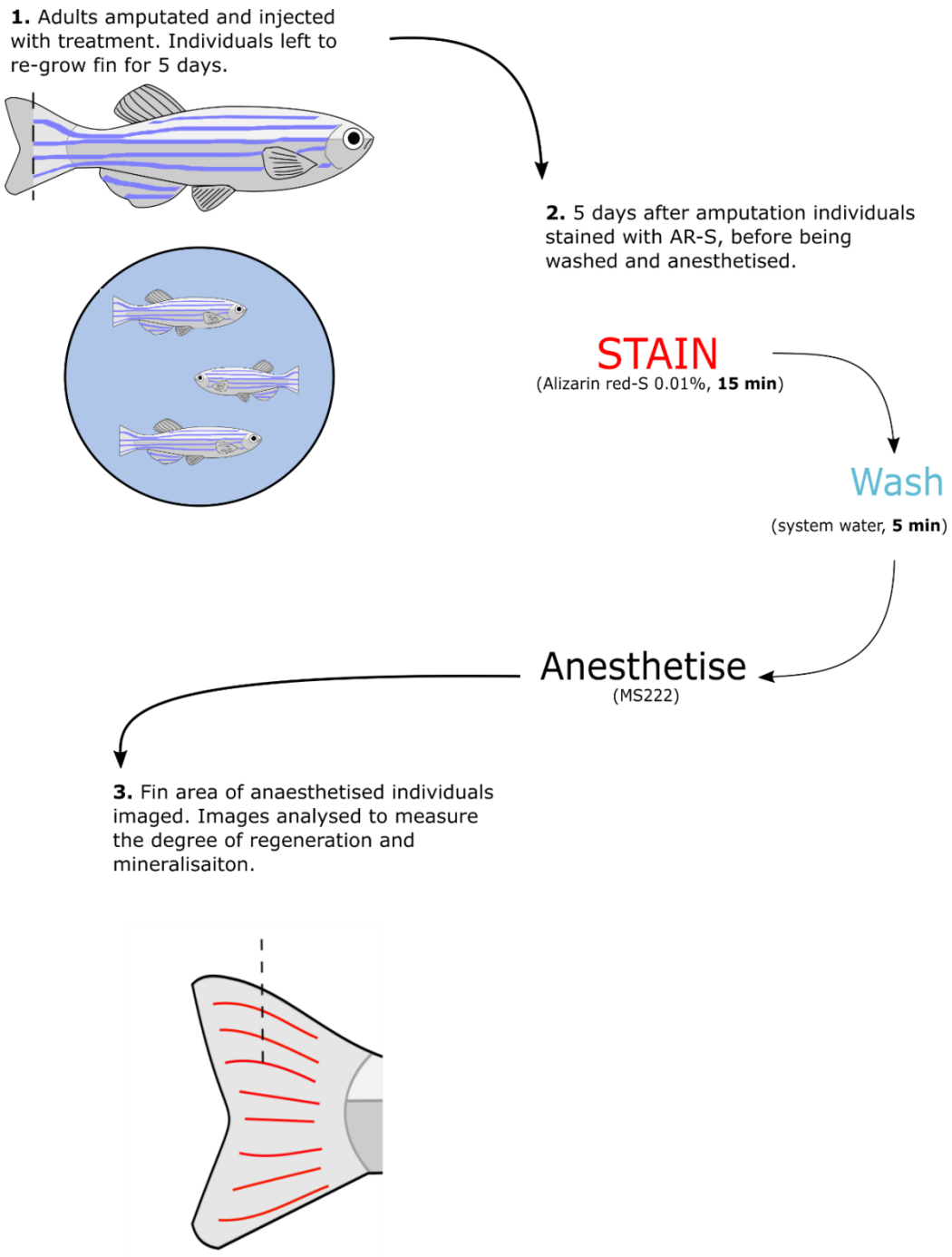


Figure 5.4: illustration depicting the caudal fin regeneration system used in adult zebrafish, to determine the *in vivo* osteogenic potential of extracts. Three key steps of the process are summarised, including the initial amputation of fins, their staining and eventual imaging.

5.2.6 Ethics statement

Experiments involving zebrafish were completed in Portugal. All experiments followed the EU Directive 2010/63/EU and related guidelines (European Commission, 2014), as well as Portuguese legislation (Decreto-Lei 113/2013) for animal experimentation and welfare. Only trained operators handled the animals and all fish facilities were accredited by the Portuguese National Authority for Animal Health (DGAV).

5.2.7 Statistical analysis

Prism version 6.00 (GraphPad Software, Inc. La Jolla, CA) software was used for statistical analysis. Datasets were tested for normality using D'Agostino-Pearson and Shapiro-Wilk test, as well as for equal variance using Brown-Forsythe test. Statistical comparisons were analysed with one-way ANOVA and post-hoc with Dunnett's multiple comparison test. Comparisons between positive controls and their relevant negative controls (operculum data) were made using unpaired t-tests with Welch's correction. For all tests, a p-value < 0.05 was considered statistically significant. One set of results, those of figure 5.20, did not have equal variance and were thus analysed via Kruskal-Wallis test. Sample sizes can be found in each figure legend. Statistical analysis was performed using Prism version 6.00 (GraphPad Software, Inc. La Jolla, CA).

As with chapter 4, when comparing treatments to control a difference will only be deemed statistically significant with p values of 0.01 or below. On figures, stars have the following meaning: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

5.3. Results

5.3.1 Operculum area system

In the main, fish tolerated the treatments well and there were only small losses in each experiment. Sample sizes could be lower than planned for one of three reasons: 1. mortality during the three-day growth period, 2. loss of fish during placement for pictures (larvae are both small and delicate, meaning this was sometimes unavoidable) and 3. excluded outliers. For mortality losses, if significant a survival plot has been included to illustrate losses. If 1-2 individuals died during testing, which was not uncommon, then a mortality plot was not included. This occurred in both control and treatment solutions – suggesting toxicity of treatments was not the sole cause of death. Fish lost during placement was a rare occurrence and was almost always limited to 1 fish/treatment group. Finally, exclusions were also rare, with only obvious outliers (confirmed by Grubbs' test) removed. Full details of all losses in each group can be found in appendix 8.

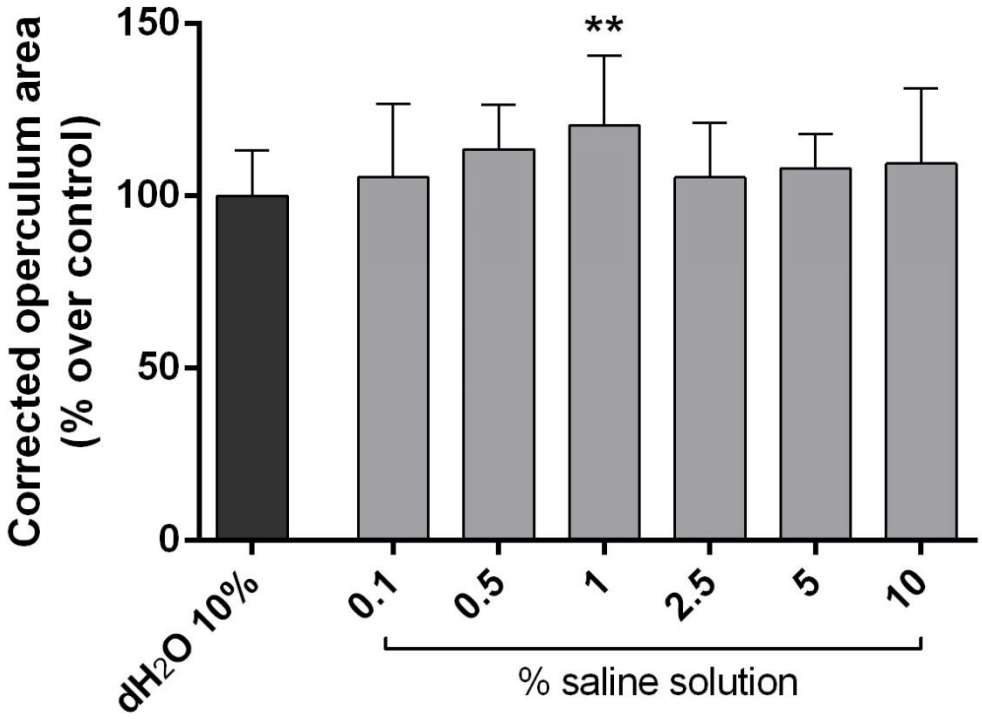


Figure 5.5: Operculum area for 10% distilled water and 0.1, 0.5, 1, 2.5, 5 or 10% saline solution treatments. Corrected operculum area is presented as mean +/- SD (n=15, 0.1% treatment n=13 – mortality). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

The safety of the vehicle controls was first tested by comparing larvae growth exposed to saline to those reared in water containing 10% distilled water (figure 5.5). Only two mortalities occurred in the 0.1% saline treatment group (14.4% of total population).

Operculum area was similar for all saline solution treatments (0.1-10%) with the exception of 1% saline treatment, which showed a small but statistically significant increase in corrected operculum area (20.5 ± 20.4%).

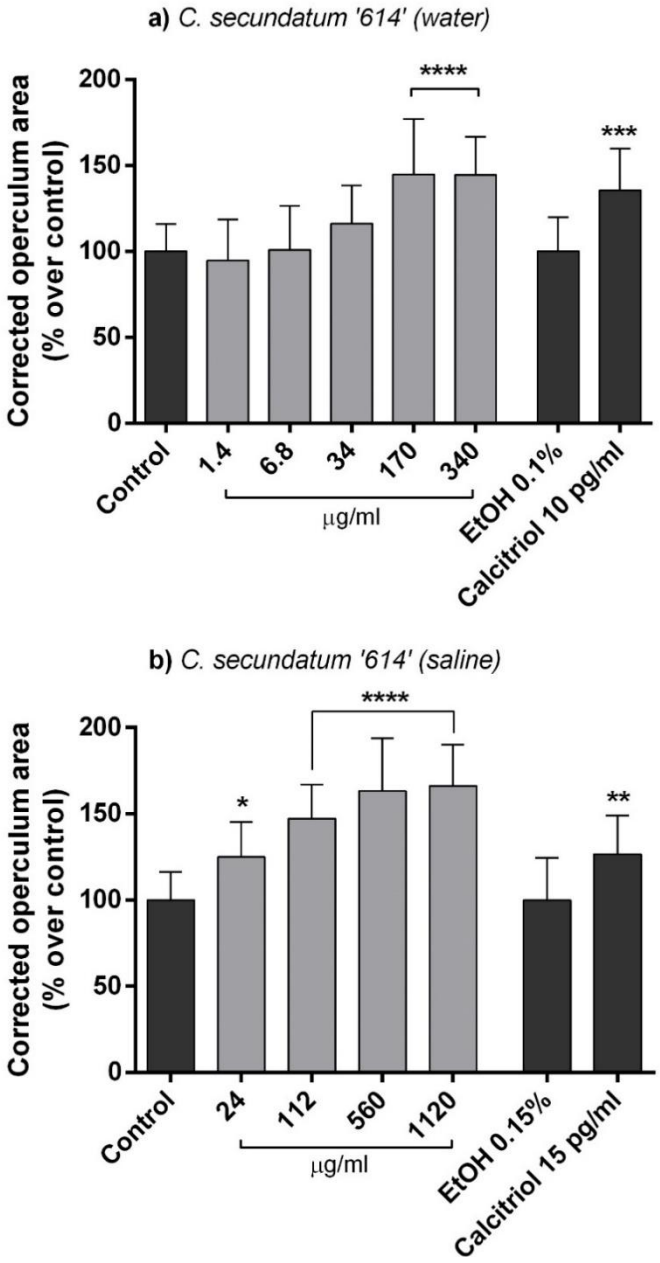


Figure 5.6: **a)** Operculum area for *C. secundatum* treatments dissolved in water. Control was system water with 10% distilled water along with a calcitriol positive control (10 pg/ml). Corrected operculum area is presented as mean +/- SD (n=13-15). **b)** Operculum area for *C. secundatum* treatments dissolved in saline solution. Control was system water with 10% saline solution. Corrected operculum area is presented as mean +/- SD (n=14-15). For each, EtOH treatment provided the vehicle control for calcitriol. Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

C. secundatum water extract caused a significant increase in corrected operculum area at 170 and 340 µg/ml concentrations, of approximately 44% [figure 5.6 a)]. 34 µg/ml treatment

increased operculum area (16.2 ± 22.2%), but not to a significant degree; whilst the two lowest treatment concentrations (1.4 and 6.8 µg/ml) were comparable in value to control. Similarly, saline treatment caused a dose-dependent increase in operculum area over the treatment period [figure 5.6 b)]. This ranged from an average increase (from control) of 24.9 ± 20.3% at the lowest concentration of 24 µg/ml, to 66.3 ± 23.8% at 1120 µg/ml. Comparing between water and saline extracts, both caused significant operculum area at their two highest tested concentrations (though overall saline extract concentrations were higher than those dissolved in water), which exceeded that seen with calcitriol treatment; though saline dissolved extract had a greater effect (maximum area increase of approximately 66% compared to 44% with water). Furthermore, saline based extract was more active than water at lower concentrations.

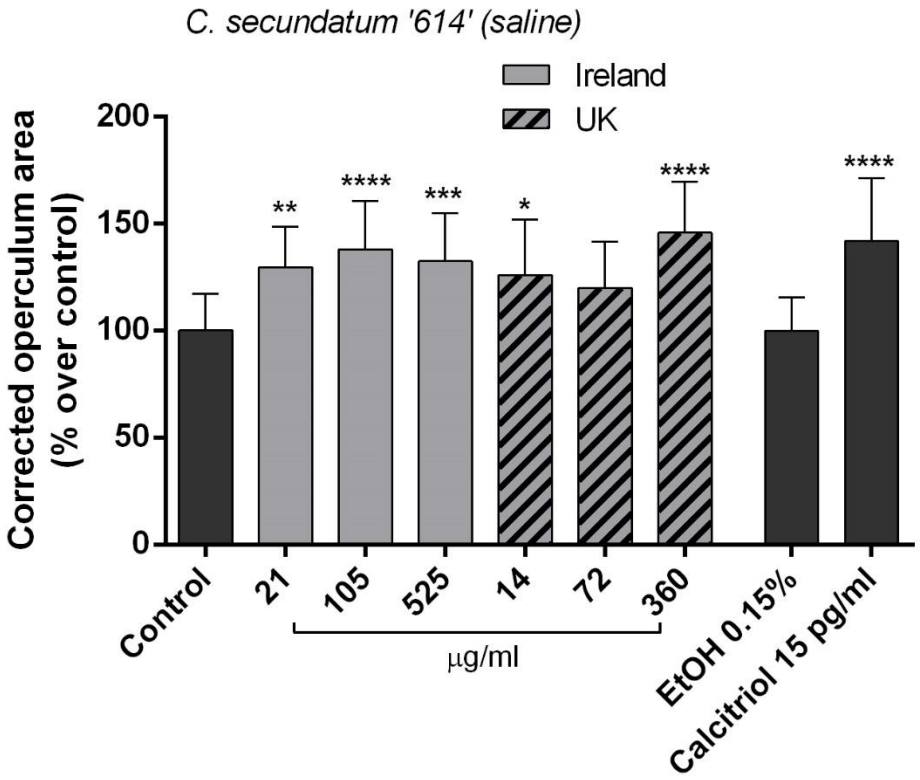


Figure 5.7: Operculum area for *C. secundatum* treatments dissolved in saline solution, from either Irish or UK-derived original sample material which was extracted 'in-house'. Control was system water with 10% saline solution. Positive control was calcitriol (15 pg/ml), with EtOH treatment as the vehicle control. Corrected operculum area is presented as mean +/- SD (n=14-15). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Ceramium secundatum Irish extract ('in-house') caused a significant increase in corrected operculum area at all tested concentrations (figure 5.7). Increases were similar in size between all treatments, with no obvious dose response. UK extract treatments had less of

an effect overall, causing small increases at 14 and 72 µg/ml concentrations, but, 360 µg/ml caused a large significant increase in operculum area of 45.8 ± 23.6%, equal to that of the calcitriol control. Both extracts from UK and Ireland produced results similar to the original extract.

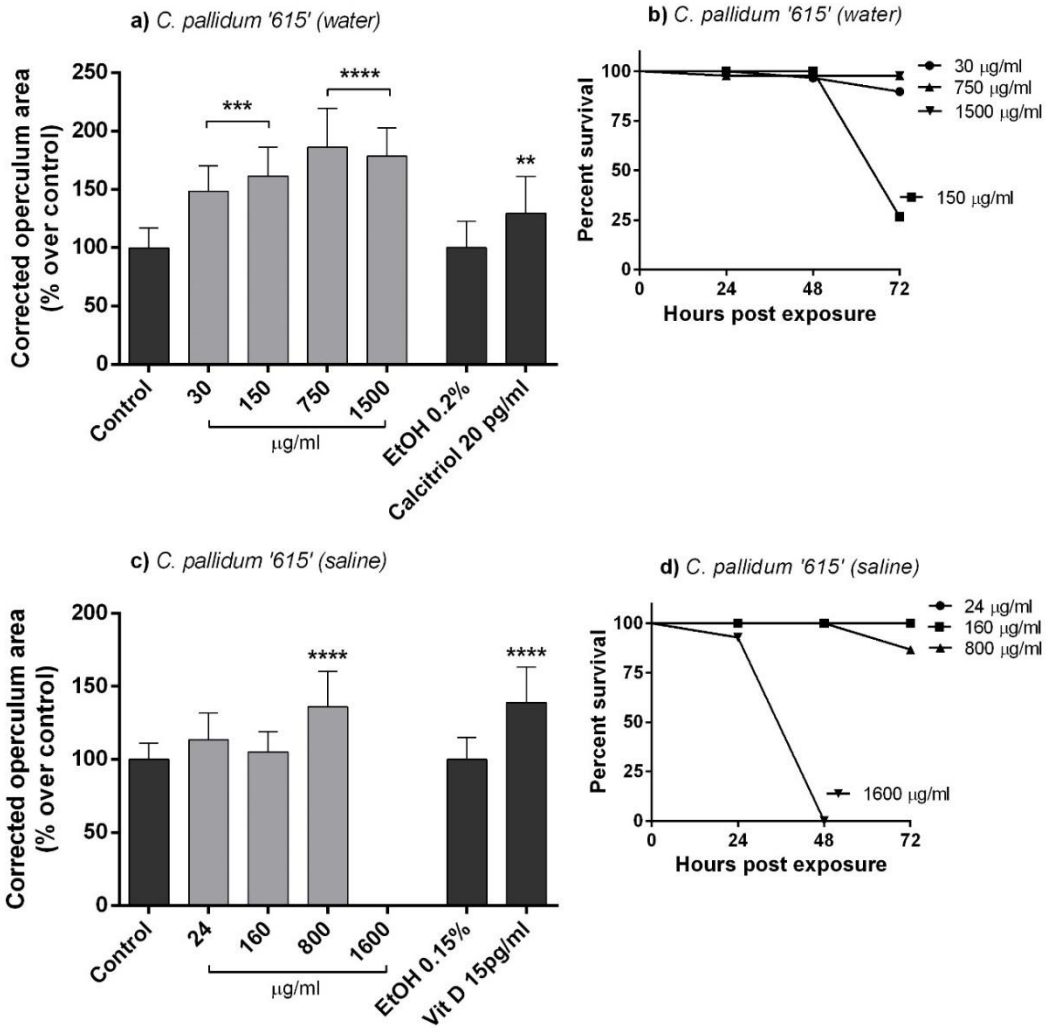


Figure 5.8: **a)** Operculum area for *C. pallidum* treatments dissolved in water. Control was system water with 10% distilled water. Positive control was calcitriol (20 pg/ml). Corrected operculum area is presented as mean +/- SD (n=4-15). **b)** Percentage survival associated with each water based treatment at either 24, 48 or 72-hour timepoints, which was notably lower at 72 hours for the 150 µg/ml treatment. **c)** Operculum area for *C. pallidum* treatments dissolved in saline solution. Control was system water with 10% saline solution. Positive control was calcitriol (15 pg/ml). Corrected operculum area is presented as mean +/- SD (n=14-15, n=0 for 1600 µg/ml). **d)** Percentage survival associated with each saline based treatment at either 24, 48 or 72-hour timepoints, which was notably lower at 48 hours for the 1600 µg/ml treatment. For each, EtOH treatment provided the vehicle control for calcitriol. Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

A significant mortality event occurred in response to both water and saline extracts of *C. pallidum* [figures 5.8 b) and d)], see discussion for explanation of this. For water extract 11/15 individuals died (73%) at 150 µg/ml concentration 72 hours post exposure, yet higher

concentrations caused no significant mortality. Conversely, saline extract caused complete mortality at the highest treatment concentration of 1600 µg/ml, 48 hours post exposure. In terms of effect on operculum area, water extract [5.8 a)] was most effective, causing significant increases at all concentrations tested. Treatment had a dose dependent effect, up to 750 µg/ml with a maximum increase of 86.1 ± 33.3% and subsequently decreasing to 78.5 ± 24.3% at 1500 µg/ml concentration. These increases were much greater than those seen with 20 pg/ml calcitriol (29.6 ± 31.6%). Comparatively, saline extract had less of an effect [5.8 c)], only causing a significant increase in operculum area at 800 µg/ml – though this was still notable (36.1 ± 24.4%).

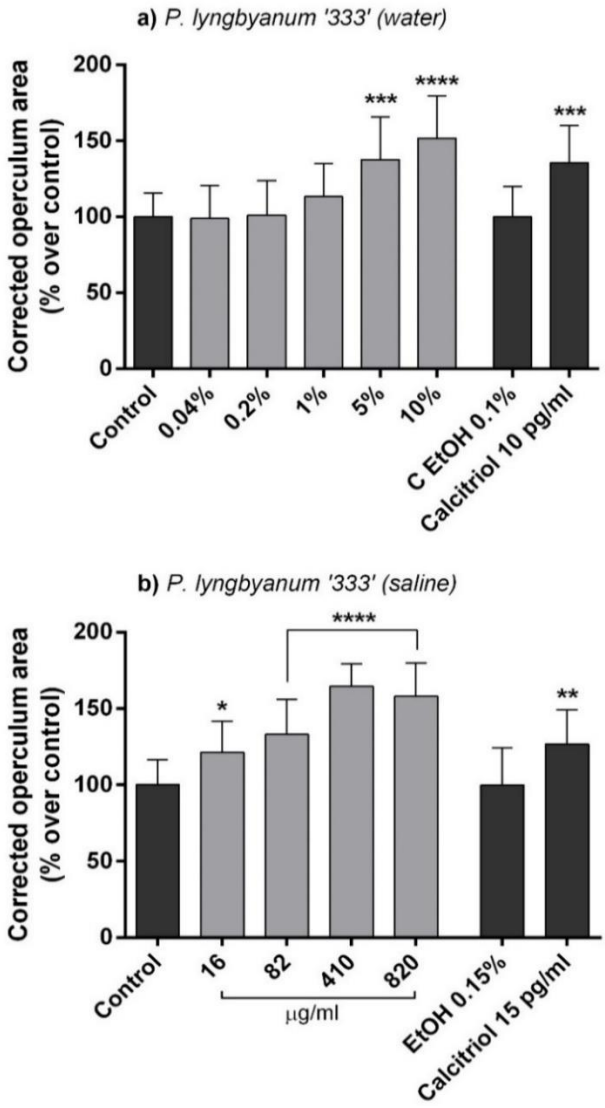


Figure 5.9: **a)** Operculum area for *P. lyngbyanum* treatments dissolved in water. Control was system water with 10% distilled water. Positive control was calcitriol (10 pg/ml). Corrected operculum area is presented as mean +/- SD (n=13-15). **b)** Operculum area for *P. lyngbyanum* treatments dissolved in saline solution. Control was system water with 10% saline solution. Positive control was calcitriol (15 pg/ml). Corrected operculum area is presented as mean +/- SD (n=13-15). For each, EtOH treatment provided the vehicle control for calcitriol. Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Plocamium lyngbyanum water extract produced less extract solution than anticipated and thus not enough was available to use for conversion of percentage values to µg/ml. *P. lyngbyanum* extract had no effect on corrected operculum area at 0.04 or 0.2% concentrations, with both being comparable to control [figure 5.9 a)]. However, 1, 5 and 10% treatments caused a dose-dependent increase in operculum area, which reached significance at 5 and 10% - with 10% treatment causing an increase of 51.7 ± 27.7%. Saline extract [5.9 b)] also had a dose dependent effect, with all treatments (excluding 16 µg/ml) significantly increased from control. These increases peaked at 410 µg/ml with a value of 64.6 ± 14.7%, decreasing slightly to 58.1 ± 21.6% at 820 µg/ml. Comparing between extracts these trends are very similar to those seen with *C. secundatum* water and saline extract treatments (figure 5.6).

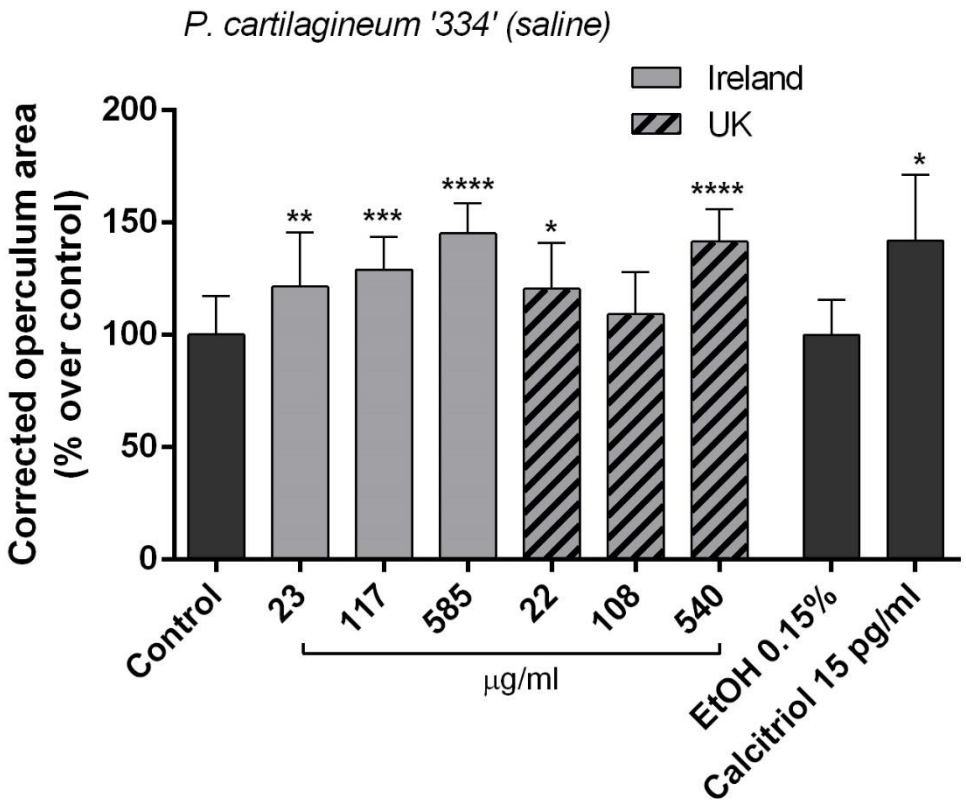


Figure 5.10: Operculum area for *P. cartilagineum* treatments dissolved in saline solution, from either Irish or UK sourced original sample material which was extracted 'in-house'. Control was system water with 10% saline solution. Positive control was calcitriol (15 pg/ml), EtOH treatment was the vehicle control. Corrected operculum area is presented as mean +/- SD (n=14-15). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

P. cartilagineum ('in-house') saline extract (figure 5.10) also showed a similar trend to that of *C. secundatum* 'in-house' saline extract (figure 5.7). For example, Irish-derived extract promoted corrected operculum area at all tested concentrations, in a dose-dependent manner. These increases were greater than those of *C. secundatum*, peaking at a maximum value of 45.1 ± 13.4% with 585 µg/ml treatment. UK extract of *P. lyngbyanum* was again very similar to the UK extract of *C. secundatum*, causing significant promotion of operculum area at 540 µg/ml concentration – though not at 22 or 108 µg/ml (9.0 ± 18.7%). Increases for maximum concentrations of both UK and Irish-derived extracts were similar in value to those seen with calcitriol (41.7 ± 29.5%). It should be noted when making comparisons between extracts that different concentration ranges were included, partly due to the amount of material which dissolved during extraction and partly based on previous *in vitro* indications of an effective concentration range. Taking UK derived 'in-house' extracts as an example, *P. cartilagineum* was tested between 22-540 µg/ml, whilst *C. secundatum* was tested between 14-360 µg/ml.

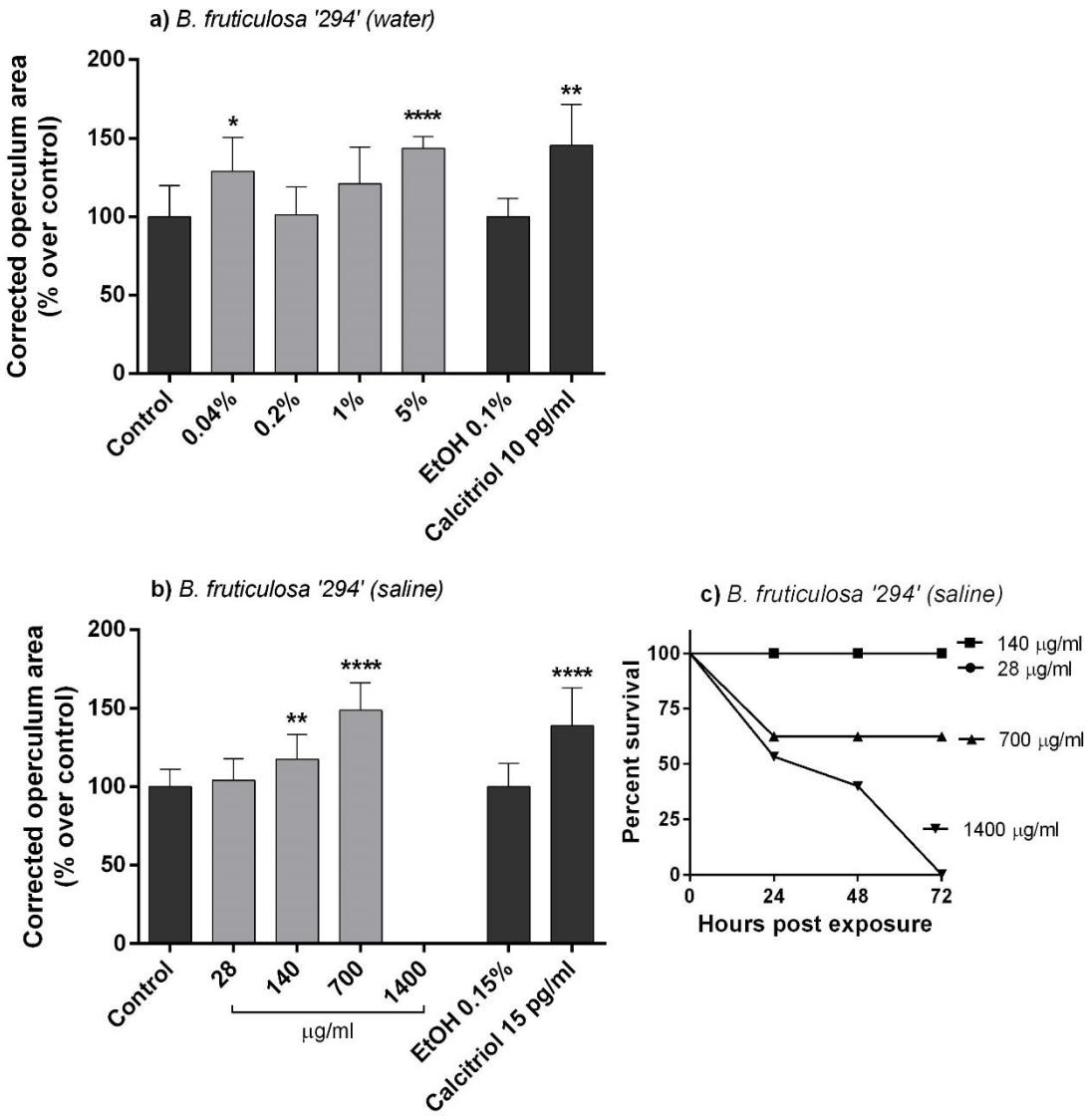


Figure 5.11: **a)** Operculum area for *B. fruticulosa* treatments dissolved in water. Control was system water with 10% distilled water. Positive control was calcitriol (10 pg/ml). Corrected operculum area is presented as mean +/- SD (n=8-10). **b)** Operculum area for *B. fruticulosa* treatments dissolved in saline solution. Control was system water with 10% saline solution. Positive control was calcitriol (15 pg/ml). Corrected operculum area is presented as mean +/- SD (n=9-15, n=0 for 1400 µg/ml). **c)** Percentage survival associated with each saline based treatment at either 24, 48 or 72-hour timepoints, which was notably decreased with 700 and 1400 µg/ml treatment. For each, EtOH treatment provided the vehicle control for calcitriol. Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Boergeseniella fruticulosa caused a dose-dependent increase in operculum area at 0.2, 1 and 5% concentrations, which reached significance (43.6 ± 7.7%) at 5% [figure 5.11 a)]. Contrary to this trend, the lowest concentration of 0.04% also increased operculum area, reaching a value of 28.8 ± 21.7%. Saline extract of *B. fruticulosa* also had a dose-dependent effect [5.11 b)], causing significant increases in operculum area of 17.6 ± 15.7% at 140 µg/ml and 48.8 ± 17.6% at 700 µg/ml. However, saline extract also had notable associated mortality, with 6

individuals (40%) dying in the 700 µg/ml treatment 24 hours after exposure. Furthermore, 1400 µg/ml *B. fruticulosa* caused complete mortality 72 hours post exposure [5.11 c)].

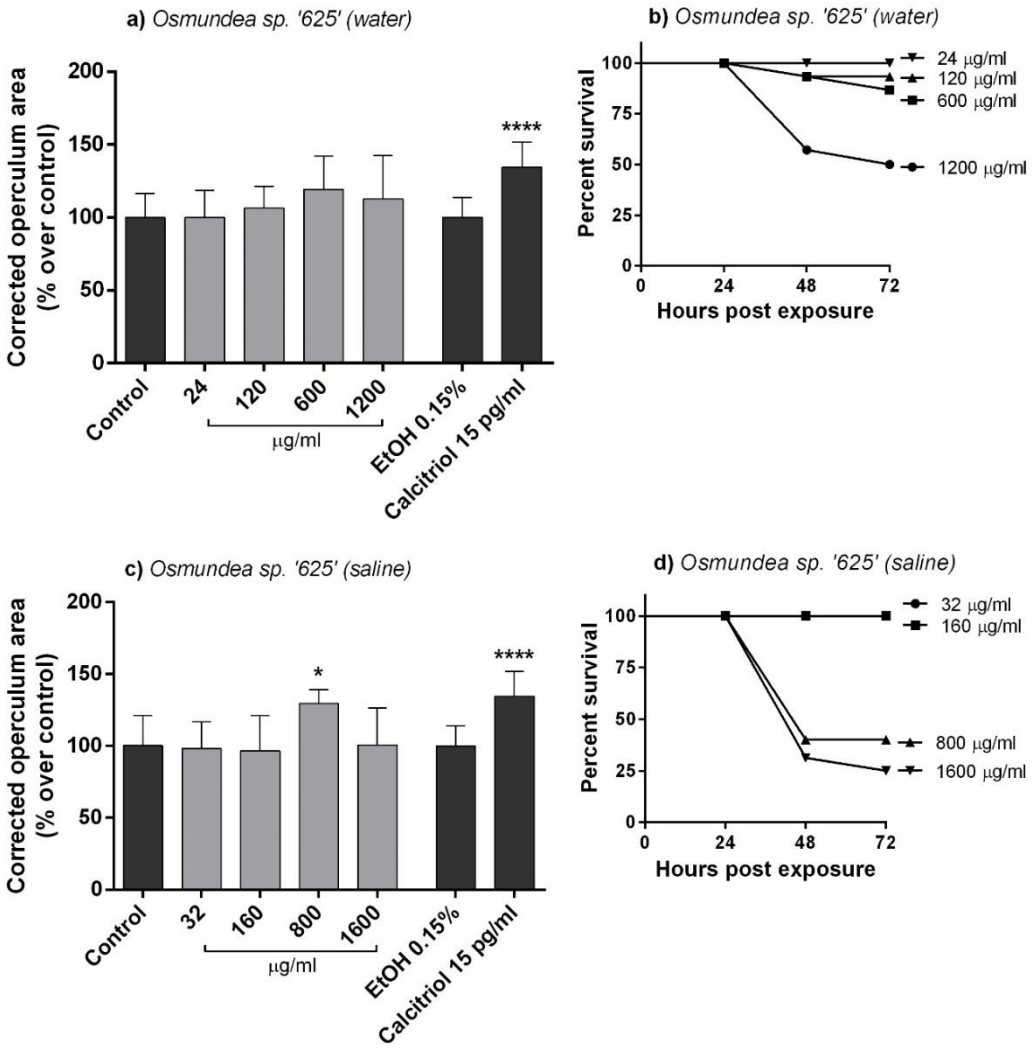


Figure 5.12: **a)** Operculum area for *Osmundea sp.* treatments dissolved in water. Control was system water with 10% distilled water. Positive control was calcitriol (15 pg/ml). Corrected operculum area is presented as mean +/- SD (n=8-15). **b)** Percentage survival associated with each water based treatment at either 24, 48 or 72-hour timepoints, which was notably reduced with 1200 µg/ml treatment. **c)** Operculum area for *Osmundea sp.* treatments dissolved in saline solution. Control was system water with 10% saline solution. A Positive control was calcitriol (15 pg/ml). Corrected operculum area is presented as mean +/- SD (n=4-15). **d)** Percentage survival associated with each saline based treatment at either 24, 48 or 72-hour timepoints, which was notably reduced for 800 and 1600 µg/ml treatments. For each, EtOH treatment provided the vehicle control for calcitriol. Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Osmundea sp. treatments had very little effect on corrected operculum area, either with water or saline extracts (figure 5.12). A slight increase was seen with 600 and 1200 µg/ml water based treatments, though these were not significant. One small increase in operculum area was seen with 800 µg/ml saline extract (29.5 ± 9.7%), though this increase was lower

than that caused by 15 pg/ml calcitriol treatment (34.6 ± 17.1%). Alternatively, mortality was notable for both extracts of *Osmundea sp*. For example, 1200 µg/ml water extract caused an approximate 50% reduction in larvae population size 72 hours post exposure. Saline extract caused an even greater decrease (74% reduction) at the maximum concentration of 1600 µg/ml, whilst 800 µg/ml was similar (60% of population died).

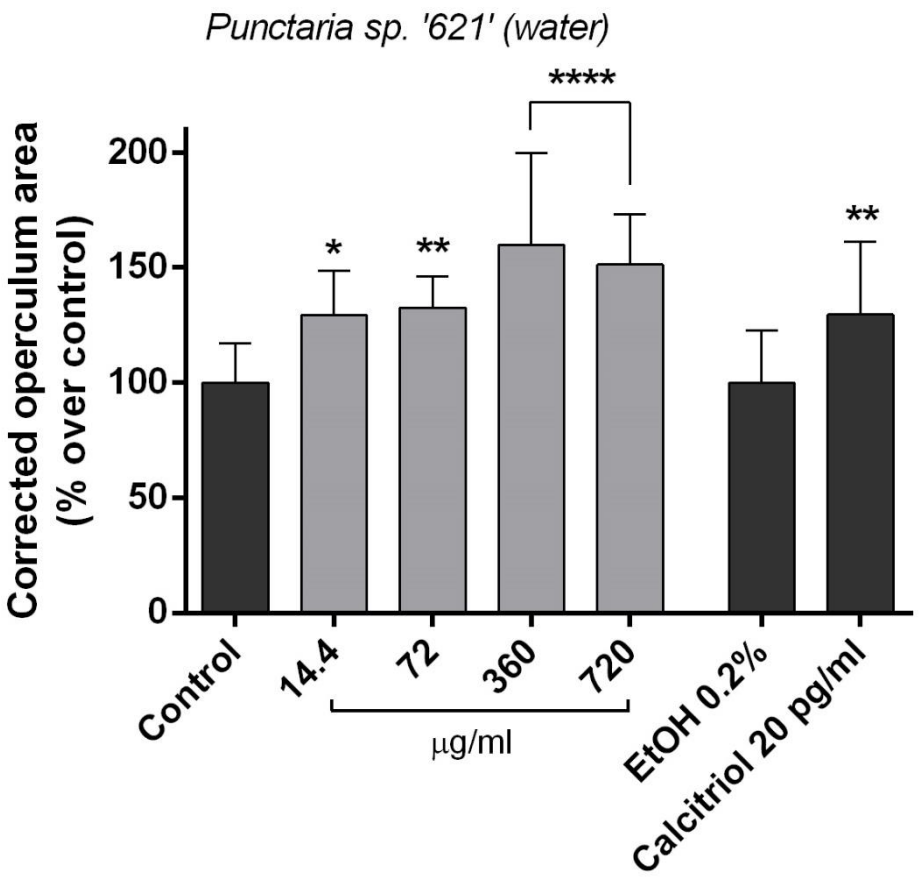


Figure 5.13: Operculum area for *Punctaria sp.* treatments dissolved in water. Control was system water with 10% distilled water. Positive control was calcitriol (20 pg/ml), EtOH treatment was the vehicle control. Corrected operculum area is presented as mean +/- SD (n=15, control n=11). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Operculum area significantly increased at concentrations of 72 µg/ml and above for *Punctaria sp.* extract (water based). 14.4 and 72 µg/ml treatments caused similar increases in size, of approximately 30%. This was also true for 360 and 720 µg/ml *Punctaria sp.* water extract, which caused an increase of approximately 55% - exceeding that of 20 pg/ml calcitriol treatment (29.6 ± 31.6%).

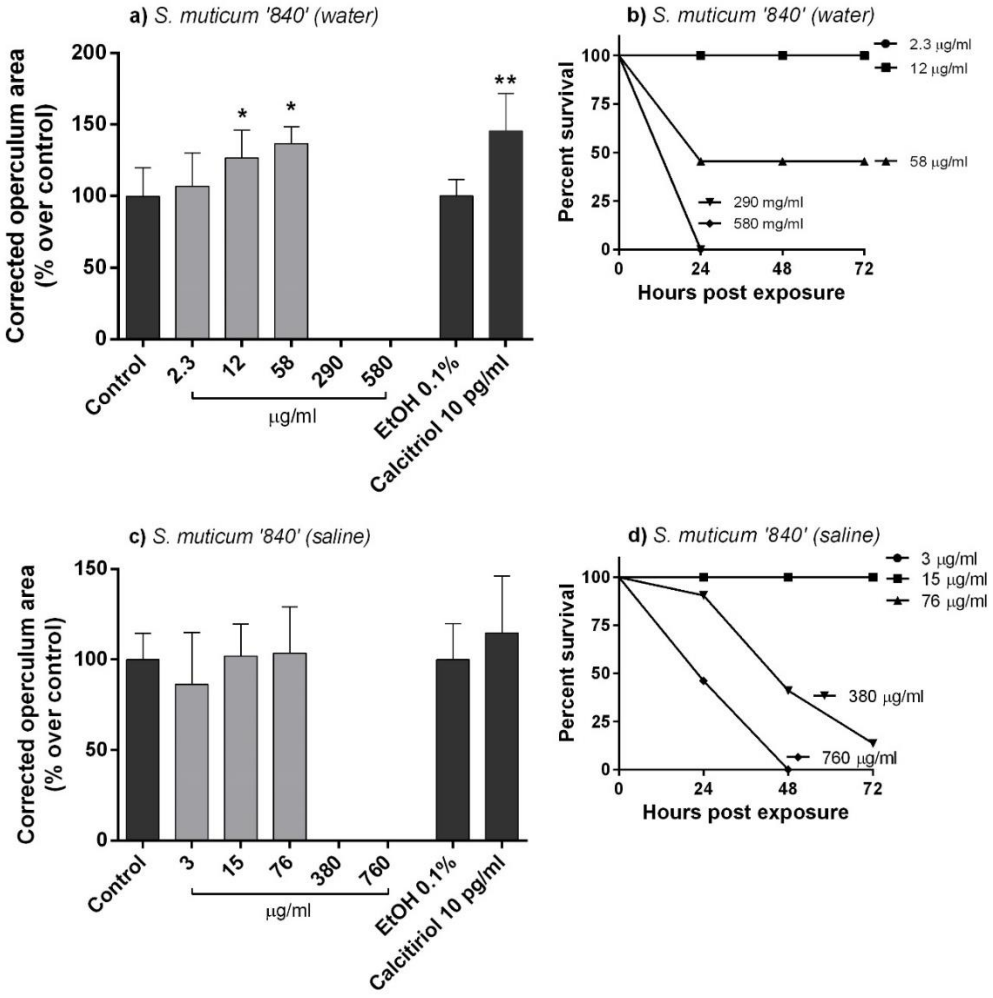


Figure 5.14: **a)** Operculum area for *S. muticum* treatments dissolved in water. Control was system water with 10% distilled water. Positive control was calcitriol (10 pg/ml). Corrected operculum area is presented as mean +/- SD (n=0-10). **b)** Percentage survival associated with each water based treatment at either 24, 48 or 72-hour timepoints. **c)** Operculum area for *S. muticum* treatments dissolved in saline solution. Control was system water with 10% saline solution. Positive control was calcitriol (10 pg/ml). Corrected operculum area is presented as mean +/- SD (n=0-10). **d)** Percentage survival associated with each saline based treatment at either 24, 48 or 72-hour timepoints. For each, EtOH treatment provided the vehicle control for calcitriol. Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Sargassum muticum water extract resulted in a dose-dependent increase in corrected operculum area, reaching a peak at 58 µg/ml – with a value of 36.7 ± 11.9% [figure 5.14 a)]. However, mortality was also significant for this treatment, with only approximately 50% of larvae surviving exposure [5.14 b)]. Furthermore, the two highest treatment concentrations – 290 and 580 µg/ml – resulted in complete mortality only 24 hours after initial exposure. Saline extract of *S. muticum* caused a decrease in operculum area of 14.7 ± 28.7% at 3 µg/ml [5.14 c)]. 15 and 76 µg/ml treatments were very similar to control and had no associated mortality. However, as with water extract treatments, higher concentrations of 380 and 760

µg/ml were highly toxic. 760 µg/ml caused complete mortality 48 hours after exposure [5.14 d)], whist with 380 µg/ml treatment only 1 larva survived to 72 hours. This larva was excluded from further analysis.

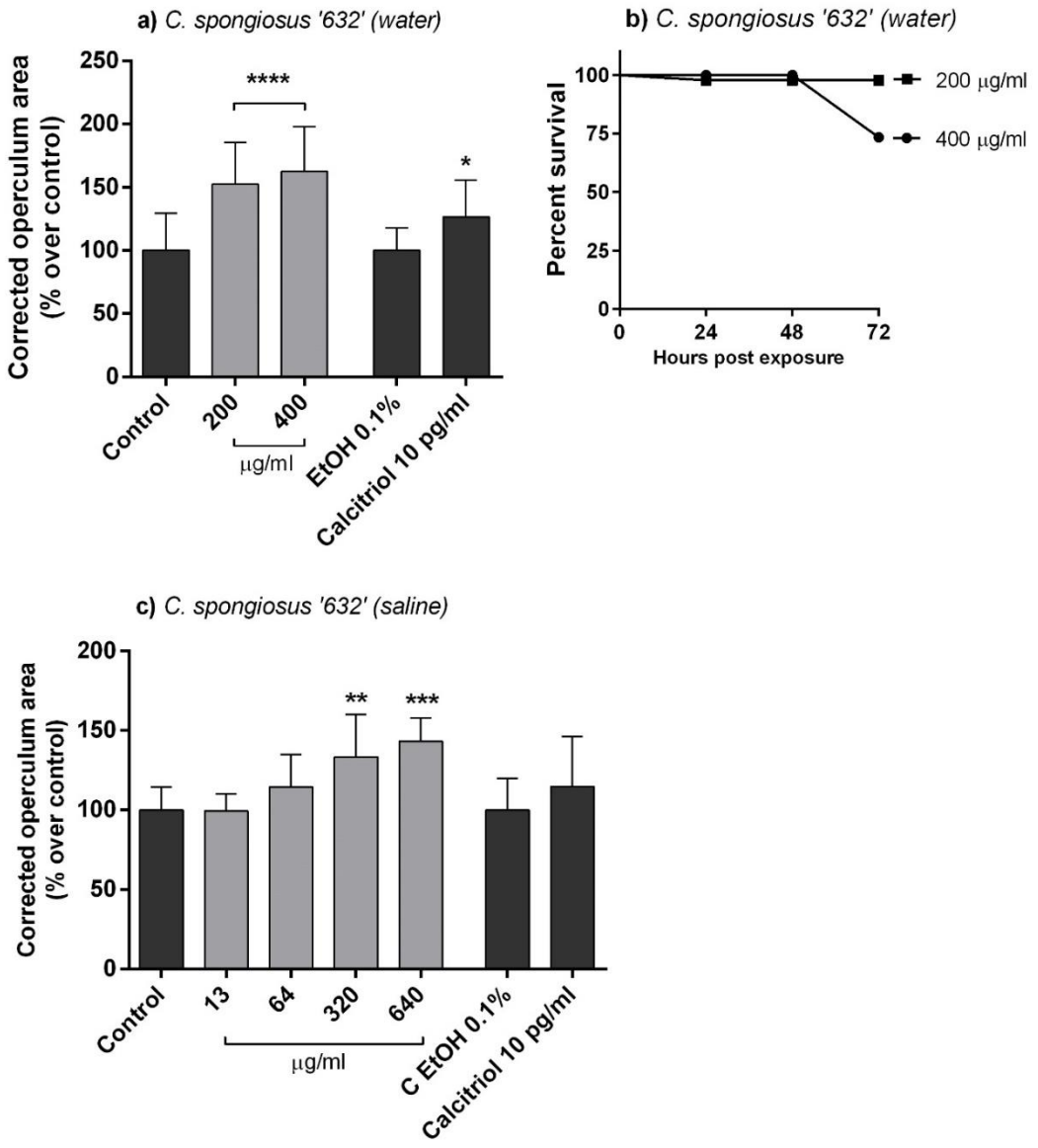


Figure 5.15: **a)** Operculum area for *C. spongiosus* treatments dissolved in water. Control was system water with 10% distilled water. Positive control was calcitriol (10 pg/ml). Corrected operculum area is presented as mean +/- SD (n=0-15). **b)** Percentage survival associated with each water based treatment at either 24, 48 or 72-hour timepoints. **c)** Operculum area for *C. spongiosus* treatments dissolved in saline solution. Control was system water with 10% saline solution. Positive control was calcitriol (10 pg/ml). Corrected operculum area is presented as mean +/- SD (n=0-10). For each, EtOH treatment provided the vehicle control for calcitriol. Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Cladostephus spongiosus water extract was tested at two concentrations – 200 and 400 µg/ml [figure 5.15 a)]. Both caused large significant increases in operculum area, though 400

µg/ml treatment was greatest – reaching a value of 62.5 ± 35.4%; exceeding that seen with 10 pg/ml calcitiriol (26.7 ± 29.1%). Of note, the 400 µg/ml treatment group also suffered 4 mortalities (26% of total population) 48-72 hours post exposure. Four concentrations of saline extract were also tested [5.15 c)], ranging in concentration between 13 and 640 µg/ml. Treatment had a dose-dependent effect on operculum area, reaching a maximum increase of 43.2 ± 14.8% at 640 µg/ml concentration – which again exceeded that seen with 10 pg/ml calcitriol (14.8 ± 31.2%).

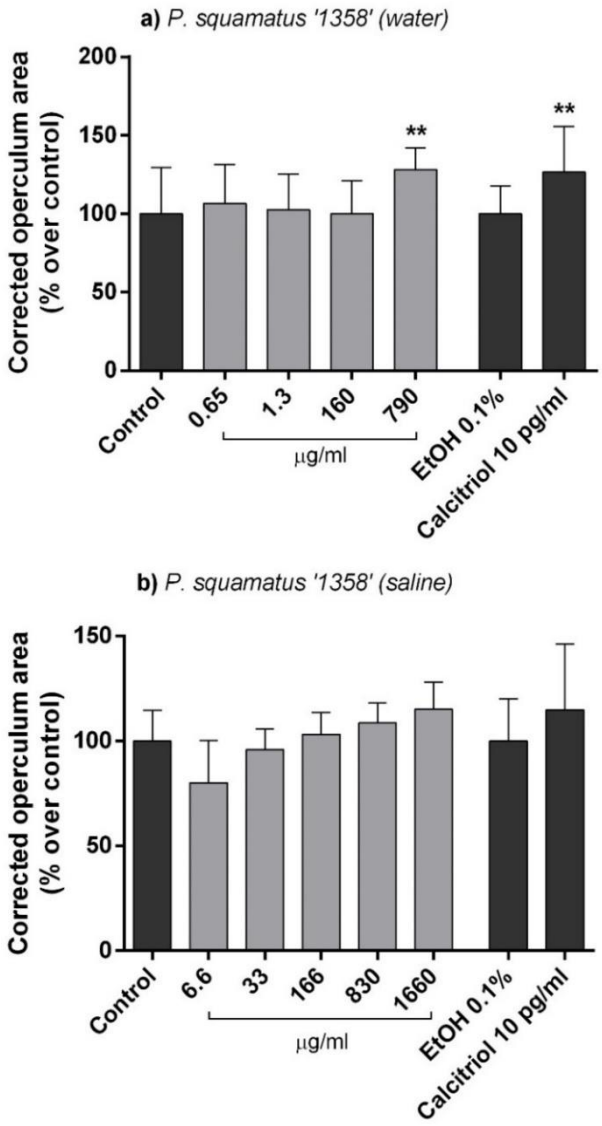


Figure 5.16: **a)** Operculum area for *P. squamatus* treatments dissolved in water. Control was system water with 10% distilled water. Positive control was calcitriol (10 pg/ml). Corrected operculum area is presented as mean +/- SD (n=14-15). **b)** Operculum area for *P. squamatus* treatments dissolved in saline solution. Control was system water with 10% saline solution. Positive control was calcitriol (10 pg/ml). Corrected operculum area is presented as mean +/- SD (n=8-10). For each, EtOH treatment provided the vehicle control for calcitriol. Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Psolus squamatus water extract had no effect on operculum growth [figure 5.16 a)] at concentrations of 0.65, 1.3 or 160 µg/ml; though 790 µg/ml treatment caused a significant 28.0 ± 14.1% increase in operculum area, similar to the increase seen with 10 pg/ml calcitriol (26.7 ± 29.1%). For saline extracts [5.16 b)], 6.6 µg/ml caused a 19.1 ± 20.1% decrease in operculum area. Subsequently, increasing extract concentration caused a dose dependent increase in operculum area, though these values were not significantly different from control – even at the maximum concentration of 1660 µg/ml. Interestingly, the positive control also didn't show a significant increase in operculum area in this experiment. Rather than being related to brood stock this was most likely a result of repeat testing using calcitriol, as it is known to degrade upon exposure to light. This means that over time higher concentrations are required to sustain effects on bone growth (i.e. 15 or 20 pg/ml concentration).

5.3.2 *In vivo* regeneration - caudal fin

Caudal fin experiments were smaller in design than those testing operculum area changes. A maximum of 6 treatment groups were included in each experiment, with 5-7 adult individuals in each group.

Both *P. squamatus* (1358) and *C. spongiosus* (632) extracts had very little effect on osteogenesis or regeneration of fins, [figure 5.17 a) and b)]. However, the higher dose of *C. spongiosus* and the single concentration tested of *P. squamatus* did cause a change in the pixel intensity analysis with a shift to the right in the distribution curve [5.17 c)]. Comparing average percentage frequency between low and high classes, both 0.25 µg/mg *C. spongiosus* and *P. squamatus* treatments had fewer low intensity pixels and more high intensity ones compared to control; though neither was significant. Alternatively, 0.13 µg/mg *C. spongiosus* was comparable to control in terms of frequency of both low and high intensity pixels.

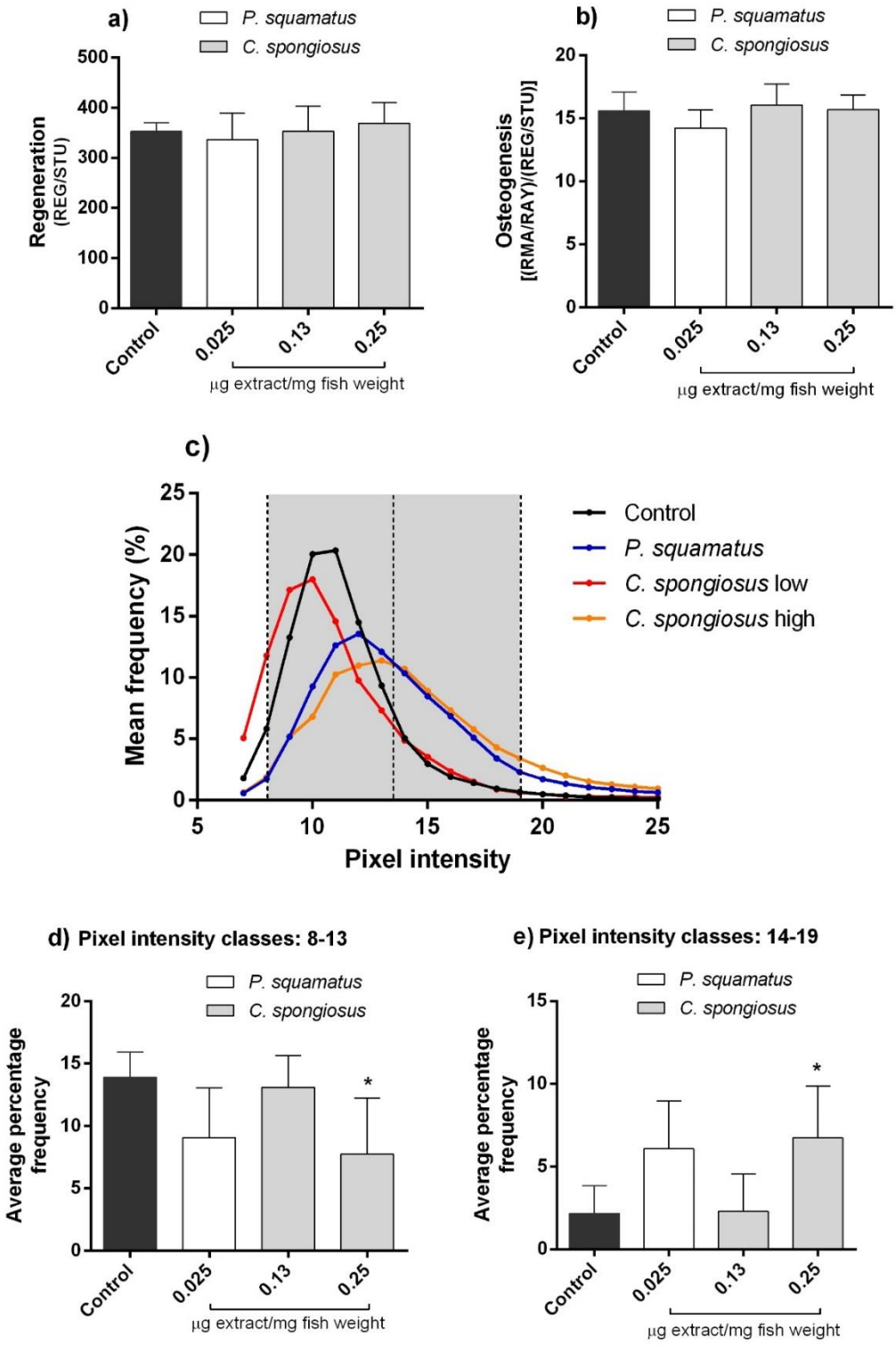


Figure 5.17: for all figures control was IP injection of distilled water 3 µl/100 mg fish weight. 3 treatments were tested, including 0.025 µg/mg (fish weight) *P. squamatus*, 0.13 and 0.25 µg/mg *C. spongiosus*, which were all dissolved in water. **a)** Degree of regeneration at 120 hpa. **b)** Degree of osteogenesis at 120 hpa. **c)** Histogram showing pixel intensity frequencies in classes 8-13 and 14-19. **d)** Average percentage frequency for control and treatments within pixel intensity classes.

of 8-13. **e)** Average percentage frequency for control and treatments within pixel intensity classes of 14-19. 1 mortality occurred after 24 hours within the control group. Results for figures **a)**, **b)**, **d)** and **e)** are presented as the mean +/- SD (n=4-7, 1 mortality in control group). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Table 5.3: mortality at 24, 48, 72, 96 and 120 hpa for control and extract treatments of 'caudal experiment 2'. Also included is a final mortality value, representing the percentage of total population that died during the 120-hour experimental period. Starting sample size was 6 for control water, control saline, *C. secundatum* (614) and *Punctaria sp.* (621). Sample size for *C. spongiosus* (632) treatments (saline and water) was 5.

Treatment	Concentration	Mortality (hours with treatment)					Final mortality
(Water)	**(µg/mg)**	**24**	**48**	**72**	**96**	**120**	**(%)**
Control water							0
614 (water)	0.51						0
621 (water)	0.93	3	1	1			83.3
632 (water)	0.25	4					80
Control saline							0
632 (saline)	1.3	4					80

In the second caudal experiment, mortality was significant for 0.93 µg/mg *Punctaria sp.* (621) and both concentrations of *C. spongiosus* (632) (see table 5.3). For each of these groups only 1 individual survived and therefore, these results have been excluded from statistical and pixel intensity analysis. Briefly, in terms of regeneration [figure 5.18 a)] *Punctaria sp.* and 0.25 µg/mg *C. spongiosus* treatments (water extracts) caused a slight increase compared to control, whilst 1.3 µg/mg *C. spongiosus* (saline extract) was reduced. Alternatively, all treatments reduced the degree of osteogenesis, though this was greatest for 1.3 µg/mg *C. spongiosus* (saline). Notably, 0.51 µg/mg *C. secundatum* (614) suffered no mortality during the 5-day experimental period, though it also showed no discernible effect on caudal fin regeneration or degree of osteogenesis. However, pixel intensity analysis was again more informative. Here, mean frequency for *C. secundatum* peaked in low intensity pixel classes (10-16) and had a much smaller distribution in higher intensity classes (17-23) [5.18 d)]. Conversely, the water control histogram was spread evenly between each class. Average percentage frequency for *C. secundatum* compared to control reflects these trends, as extract treatment caused a significant increase in frequency of low intensity pixels and a significant reduction in high intensity ones.

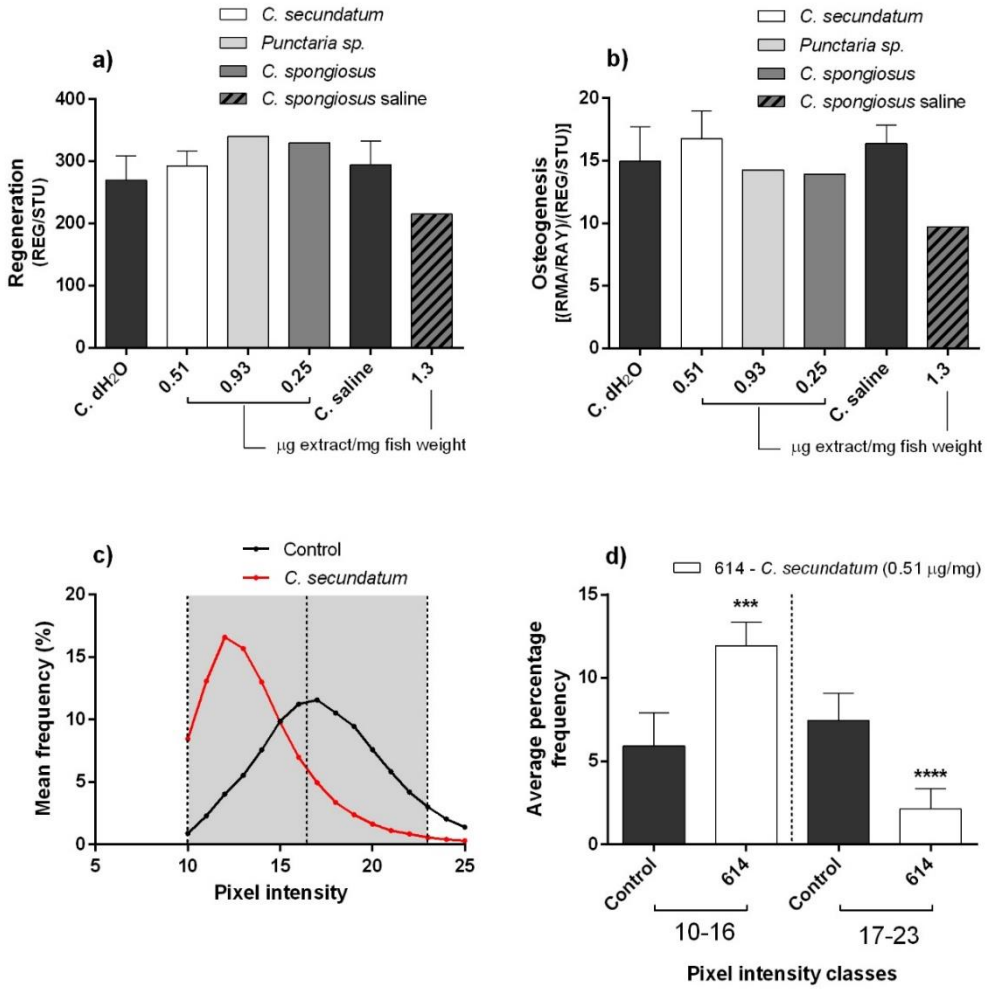


Figure 5.18: for all figures control was either IP injection of distilled water (3 µl/100 mg fish weight) (C. Dh_2O) or saline solution (C. saline) and 4 treatments were tested. These included 0.51 µg/mg (fish weight) *C. secundatum*, 0.93 *Punctaria sp.* and 0.25 *C. spongiosus* dissolved in water, as well as 1.3 µg/mg *C. spongiosus* in saline solution. **a)** Degree of regeneration at 120 hpa. **b)** Degree of osteogenesis at 120 hpa. **c)** Histogram showing pixel intensity frequencies in classes 10-16 and 17-23. **d)** Average percentage frequency for control and treatments within pixel intensity classes of 10-16 and 17-23. Mortality was significant for *Punctaria sp*. and both *C. spongiosus* treatments – see associated mortality table. Results for figures **a)**, **b)** and **d)** are presented as the mean +/- SD (n=1-6). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

In the final caudal experiment, all extracts tested had little effect on regeneration or osteogenesis (figure 5.19), with all deviations from control being small and non-significant.

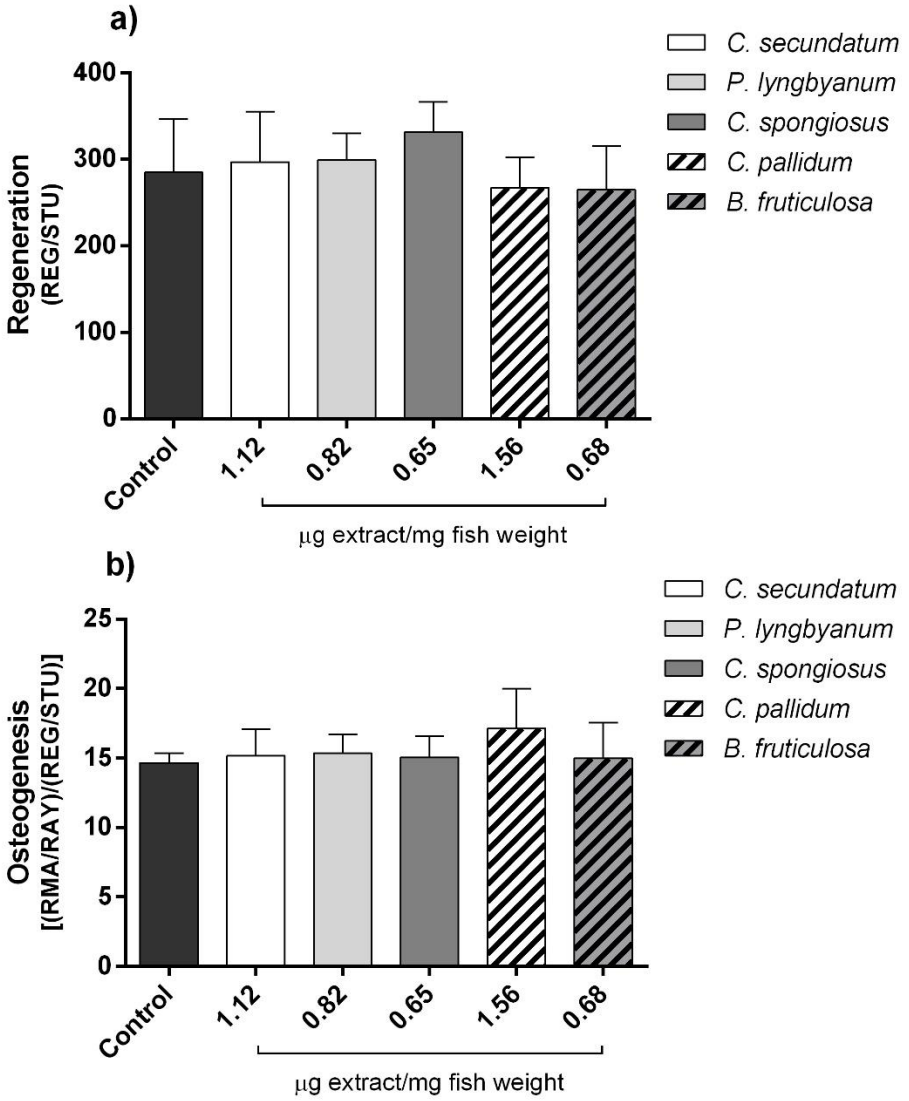


Figure 5.19: for both figures control was IP injection of saline solution (3 µl/100 mg fish weight), and 5 treatments were tested. These included 1.12 µg/mg (fish weight) *C. secundatum*, 0.82 µg/mg *P. lyngbyanum*, 0.65 µg/mg *C. spongiosus,* 1.56 µg/mg *C. pallidum* and 0.68 µg/mg *B. fruticulosa* in saline solution. **a)** Degree of regeneration at 120 hpa. **b)** Degree of osteogenesis at 120 hpa. Results are presented as the mean +/- SD (n=5, n=4 for *C. spongiosus* due to 1 mortality).

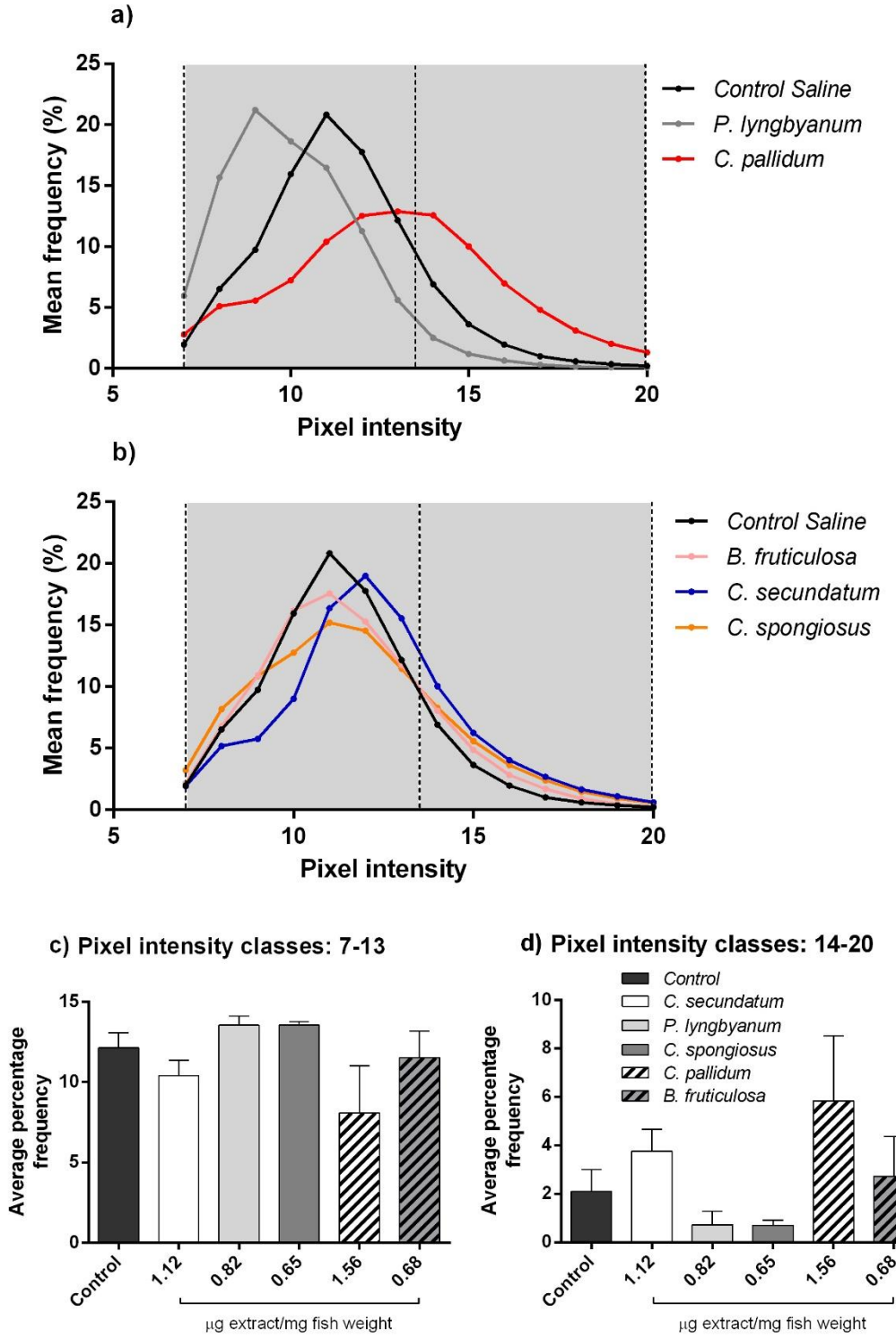


Figure 5.20: for all figures control was IP injection of saline solution (3 µl/100 mg fish weight), and 5 treatments were tested. These included 1.12 µg/mg (fish weight) *C. secundatum*, 0.82 µg/mg *P. lyngbyanum*, 0.65 µg/mg *C. spongiosus,* 1.56 µg/mg *C. pallidum* and 0.68 µg/mg *B. fruticulosa* in saline solution. **a) b)** Histogram showing pixel intensity frequencies in classes 7-13 and 14-20. **c)** Average percentage frequency for control and treatments within pixel intensity classes of 7-13 and **d**) 14-20. Results for figures **c)** and **d)** are presented as the mean +/- SD (n=5, n=4 for *C. spongiosus* due to 1 mortality). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and control.

Mean frequency of control treatment peaked within the low intensity pixel class (7-13), and therefore had a much smaller distribution within the high intensity class. *P. lyngbyanum* (333) shared this trend, though its peak was at an even lower frequency than that of control. Contrary to this, *C. pallidum* (615) had a more even distribution and comparatively a much greater mean frequency within high intensity classes. Remaining extracts [*B. fruticulosa* (294)*, C. secundatum* (614) and *C. spongiosus* (632)] gave pixel distributions similar to each other and to control. Average percentage frequency data [figure 5.20 c) and d)] showed some differences between treatments and control. For low intensity classes *C. secundatum* and *C. pallidum* reduced the frequency. *C. pallidum* also had a greater average percentage frequency than control within high intensity classes. Other effects were also evident, such as an increase for *C. secundatum* and two reductions for *P. lyngbyanum* and *C. spongiosus* treatments – though despite their apparent size these were not statistically significant.

Table 5.4: summary of each extracts performance, highlighting whether they had a stimulatory or toxic effect in the operculum area and caudal fin regeneration systems.

	Extract ID	**Species**	**Stimulatory effect?**	**No significant mortality?**
Operculum area	614 (w)	*Ceramium secundatum*	✓	✓
	614 (s)		✓	✓
	614 (s) 'in-house'		✓	✓
	615 (w)	*Ceramium pallidum*	✓	×
	615 (s)		✓	×
	333 (w)	*Plocamium lyngbyanum*	✓	✓
	333 (s)		✓	✓
	333 (s) 'in-house'	*Plocamium cartilagineum*	✓	✓
	294 (w)	*Boergeseniella fruticulosa*	✓	✓
	294 (s)		✓	×
	625 (w)	*Osmundea sp.*	×	×
	625 (s)		×	×
	621 (w)	*Punctaria sp.*	✓	
	840 (w)	*Sargassum muticum*	×	×
	840 (s)		×	×
	632 (w)	*Cladostephus spongiosus*	✓	✓
	632 (s)		✓	✓
	1358 (w)	*Psolus squamatus*	✓	✓
	1358 (s)		×	✓

Caudal Fin Regeneration	1358 (w) [0.025 µg/ml]	*Psolus squamatus*	×	✓
	632 (w) [0.13 µg/ml]	*Cladostephus spongiosus*	×	✓
	632 (w) [0.25 µg/ml]	*Cladostephus spongiosus*	×	✓
	614 (w) [0.51 µg/ml]	*Ceramium secundatum*	×	✓
	621 (w) [0.93 µg/ml]	*Punctaria sp.*	×	×
	632 (w) [0.25 µg/ml]	*Cladostephus spongiosus*	×	×
	632 (s) [1.3 µg/ml]		×	×
	614 (s) [1.12 µg/ml]	*Ceramium secundatum*	×	✓
	333 (s) [0.82 µg/ml]	*Plocamium lyngbyanum*	×	✓
	632 (s) [0.65 µg/ml]	*Cladostephus spongiosus*	×	✓
	615 (s) [1.56 µg/ml]	*Ceramium pallidum*	✓	✓
	294 (s) [0.68 µg/ml]	*Boergeseniella fruticulosa*	×	✓

5.4 Discussion

This chapter's purpose was to test the *in vivo* potential of powder extracts using two zebrafish systems, one determining operculum bone formation in larval zebrafish and the other assessing caudal fin regeneration in adults. It was hoped that these results would complement previous *in vitro* data, whilst giving a better indication of those extracts with the best osteogenic and preclinical potential.

5.4.1 Operculum area system

5.4.1.1 Operculum system design and benefits

The operculum, or gill bone, is a flat bone that sits near the surface of the skull; making it easy to image. Over time, the operculum becomes dermally ossified, covering the gills of adult fish and making up (along with 28 other bones) the outer covering of the skull (Nüsslein-Volhard & Dahm 2002). In larvae, the operculum is one of the earliest cranial bones to ossify, being visible via alizarin red-S staining at only 3 dpf (Kimmel et al. 2010). Additionally, zebrafish larvae have translucent bodies, further aiding imaging of operculae and other bones. As such, operculum staining is a viable option for determining the degree of bone growth in larvae, which are relatively inexpensive to work with and (due high fecundity), easy to source. This model was first detailed in a study by Tarasco et al. (2017), which showed the operculum to be sensitive to both pro and anti-osteogenic effects caused by calcitriol and cobalt chloride treatments. Results using this system were highly repeatable and - due to its short three-day experimental period – allowed for rapid and high throughput determination of *in vivo* osteogenic potential. Other similar methods used to screen for osteogenicity have been described, such as that of Luo et al. (2016) which determined mineralisation in larval cranial bones. However, this method and others like it do not account for inter-specimen size variability, which is often significant and can impact results – as larger individuals also have larger bones, leading to false positives. The present operculum system used in this work accounts for this, through normalisation of operculum area to head area measurements, and is therefore more robust.

The most important benefit of using this model for the current project was the low levels of extract material required for testing, despite its use of waterborne exposure instead of intraperitoneal injection. This was because 6 well plates used to house larvae only required 10 ml of treatment water, equating to relatively small amounts of extract material even at the highest test concentrations. As previously described, powder extracts had more material

available for use than those dissolved in ethanol and DMSO, but not an exhaustive amount. This system therefore allowed experiments to be completed without depleting extract stocks.

5.4.1.2 Extract effects on operculum area

Previous *in vitro* work, with some exceptions such as *Punctaria sp*. (621) extract, tested extracts dissolved in saline solution – rather than water. Upon starting *in vivo* testing there was a concern that required saline concentrations during treatment (of up to 10% total volume) would lead to increased larval mortality, as zebrafish are a freshwater species. As such, water extracts of *P. squamatus* (1358) and *C. spongiosus* (632) were used for initial testing, which also included a preliminary test of saline solutions between 0.1 and 10% concentrations. Contrary to initial concerns, zebrafish survived saline concentrations of up to 10% with no significant change in mortality rates. Of note, 1% saline solution did cause a small significant increase in operculum area. However, this effect was isolated and was not seen at higher or lower concentrations, suggesting it was an anomaly. Overall, this suggested saline extracts should be tested, though water based treatments had also shown potential to increase operculum area in preliminary tests. Therefore, it was decided that future work would test both saline and water based extracts.

Overall, powder extracts frequently caused large increases in operculum area over the three-day experimental period. Red algae are an excellent example of this, as extracts from multiple species caused notable increases in operculum area – both when dissolved in water and saline solution. For example, high concentrations of water and saline dissolved *C. secundatum* (614) extract caused increases in operculum area that exceeded those seen with calcitriol. Calcitriol - the bioactive form of vitamin D - was used as a positive control, as it is known to enhance operculum growth (Tarasco et al. 2017) and the development of other skeletal structures (Fleming et al. 2005). Both extracts outperformance of calcitriol therefore demonstrates their excellent ability to promote bone growth, indicating higher mammals may also show an osteogenic response to treatment. Of note, lower concentrations of water based *C. secundatum* extract had less of an effect than comparable concentrations of saline extract; indicating a different bioactive may be present in each, or water extract may contain a lower bioactive concentration. Comparing to *in vitro* data (chapter 4), original *C. secundatum* extract caused increased hBMSC proliferation, particularly at 350 µg/ml, whilst 70 µg/ml promoted cell differentiation – though less of an effect was seen in terms of

mineralisation. These results, coupled with the described operculum area increase, indicate that *in vitro* effects are being maintained *in vivo*. As such, larval bone formation may have been stimulated through increased osteoblast number and/or differentiation. One relevant study showed a similar effect of tashinol, a polyphenolic compound from the root of Chinese sage, *Salvia miltiorrhiza* Bunge (Luo et al. 2016). Here, water extracts containing tashinol were shown to stimulate zebrafish bone formation and bone mass, as determined through fluorescence intensity after AR-S staining. This effect was thought to be due to the antioxidant capacity of tashinol, as the compound helped protect bone formation whilst larvae were exposed to oxidative stress. Similar work also described the ability of deferoxamine, an iron chelator, to remove iron and reduce oxidative stress in iron-overloaded larvae – helping to preserve the degree of bone formation (Chen et al. 2014).

'In-house' *C. secundatum* (614) was expected to have a comparable effect to original powder material, but, although significant increases in operculum area were still seen, the overall effect was reduced. This could be due to changes in brood stock, as these extracts were tested at the end of the 4-month experimental period; at which point a younger (and more fecund) breeding couple were being used for egg production. Eggs from this couple tended to hatch larvae that showed smaller changes in operculum area, even upon calcitriol treatment. Alternatively, it reflects a difference in extract activity, potentially due to differences in bioactive composition or concentration. Furthermore, there are a range of other variables to be considered between original and fresh ('in-house') extract material (as mentioned in chapter 4); including geographical variation, seasonality effects, differences in sampling location conditions and small variations in extraction method. Comparing to *in vitro* data, these results are less surprising, as both Irish and UK-derived 'in-house' extracts had less of an effect on cell proliferation than original *C. secundatum* material. However, mineralisation levels with 'in-house' extracts were much greater than those seen with original powder extract. This could potentially indicate that an increase in osteoblast proliferation is most important to stimulate early bone formation in larvae. Overall, given the difference in brood stock used to test original and 'in-house' extracts, *in vivo* activity between these extractions appears to be repeatable. *In vitro*, activity between extractions was not repeatable, though both still stimulated separate measures of cell activity (indicating bioactive presence).

Another species of the *Ceramium* genus, *C. pallidum* (615), also caused a large increase in operculum area. Saline extract likely contained a different bioactive compared to water extract, or lower concentrations of active compounds/molecules – explaining why its effect

was smaller and limited to one concentration. Furthermore, mortality at 1600 µg/ml indicated that saline extract contained different constituents to those of water, that were toxic at this concentration. Alternatively, changes in operculum area upon water extract treatment were particularly notable, with the maximum increase of 86.1 ± 33.3% the greatest pro-osteogenic effect seen using this system to date. As with *C. secundatum* extracts, this indicates a strong promotion of *in vivo* mineralisation and therefore good pre-clinical potential, particularly at high concentrations. Higher concentrations regularly performed better in the operculum system, which was not unexpected - given that treatments were applied systemically and had to adsorb through the skin or be ingested via swallowing (Zhang et al. 2015). Of note, one large mortality event did occur 72 hours post treatment in 150 µg/ml water extract group. Whilst concerning, this event is unlikely to indicate extract toxicity, considering it was limited to one treatment well and not seen when individuals were exposed to higher concentrations. Comparing to *in vitro* data, *C. pallidum* extracts greatest impact was on hBMSC mineralisation, as well as one significant increase in day 7 ALP activity. Therefore, operculum activity may be supported by potent bioactives able to stimulate cell maturation and eventual mineralisation levels – which would explain the increase in operculum area described. Related work, also utilising the zebrafish operculum system, showed that crude extracts from the green macroalgae *Cladophora rupestris* and *Codium fragile* stimulated operculum area increases (Surget et al. 2017). These effects were similar but just smaller than those of *C. pallidum*, with each macroalgae causing an approximate 75% increase in operculum area – as opposed to the 86% of the current study.

P. lyngbyanum's (333) promotion of operculum area was very similar to that of *C. secundatum* (614), for both water and saline extracts – with saline performing better overall but water promoting operculum area at the two highest concentrations. This suggests extracts from each species contain bioactives which are similar in their *in vivo* effect and ability to promote bone growth. However, it is unlikely that extracts from both species contain identical bioactives, based on differences in their *in vitro* activity profiles. For instance, like *C. secundatum*, *P. lyngbyanum* promoted hBMSC activity, though its effect was greatest on cell proliferation and mineralisation – rather than differentiation. 'In-house' extract of *P. cartilagineum* again had very similar activity to that of *C. secundatum* ('in-house'), causing significant increases in operculum area; though not to the same degree as those seen with original extract material. Again, this is could have been impacted by the change to a new brood stock or extract based variables such as sampling location, extraction procedure and interspecies differences between *P. lyngbyanum* and *P. cartilagineum* (as

previously detailed, *P. cartilagineum* was the closest match to *P. lyngbyanum* present at sample sites, and thus was used as a replacement).

B. fruticulosa (extract 294) caused a smaller stimulation of operculum area than *C. secundatum*, *C. pallidum* and *P. lyngbyanum* extracts. However, increases at the highest concentrations (5% water extract and 700 µg/ml saline) were still significantly increased from control and comparable to those of calcitriol. One increase, with 0.04% water treatment, appears to be an outlier or anomalous result, as higher concentrations of 0.2 and 1% had a smaller effect on operculum area. As with *C. pallidum*, saline extract of *B. fruticulosa* likely contains a molecule or compound which is toxic at high concentrations, explaining the mortality seen within the 1600 µg/ml treatment group. Comparing to *in vitro* data, *B. fruticulosa* had no toxic effect and instead reduced hBMSC proliferation at its highest test concentration (470 µg/ml), but promoted differentiation and mineralisation; particularly at high concentrations. This could therefore indicate that increases in operculum area are stimulated upon extract treatment due to an increase in cell maturation and degree of mineral production.

Water and saline dissolved extracts of *Osmundea sp.* (625) were an exception to the previously described activity of red algae, having a limited effect on operculum growth. Only 800 µg/ml saline extract promoted operculum area, whilst higher extract concentrations of both extracts had moderate-large toxic effects. This indicates the presence of material which is toxic at higher concentrations, which could – particularly for saline extracts – mask osteogenic effects of other molecules/compounds. This limited activity is not surprising though, as *Osmundea sp.* extract had very little *in vitro* effect on either proliferation, differentiation or mineralisation and was specifically brought forward for *in vivo* testing as a negative control. Its lack of effect therefore supports previous *in vitro* results and the robustness of the operculum area system for determining osteogenic effects.

Remaining extracts included those from the brown algae's *Punctaria sp.* (621), *S. muticum* (840) and *C. spongiosus* (632), as well as the deep-sea sponge *P. squamatus* (1358). *Punctaria sp.* was only tested as a water extract, as saline dissolved material was not included during *in vitro* testing. It had a similar activity profile to red algaes such as *C. secundatum* and *P. lyngbyanum* indicating it also contains an osteogenic bioactive(s), which is particularly effective at higher concentrations. Furthermore, *in vitro* results again support *in vivo* findings, as *Punctaria sp.* extract promoted all cell activity measures – proliferation, differentiation and mineralisation – to varying degrees over a range of tested concentrations. Related *in vivo*

work also showed another brown algae extract, a hot water extraction by-product from *Hizikia fusiforme*, to stimulate zebrafish bone formation (Jeong et al. 2016). However, it should be noted that this study only carried out qualitative analysis of stained bones and therefore its results are less robust than those of the current study. Continuing with the current work, *S. muticum* (another negative control extract) showed much less activity than *Punctaria sp*., being similar to *Osmundea sp.* extract in its effect and actually decreasing operculum area at lower concentrations. Furthermore, increases in operculum area seen with water extract were small (lower than those produced by calcitriol), whilst higher concentrations of both water and saline extract were toxic. However, unlike *Osmundea sp.* extract, *S. muticum* produced complete mortality at higher concentrations; indicating the presence of more potent toxic constituents or a greater concentration of weaker molecules. *In vitro*, reduced cell growth and differentiation seen with high *S. muticum* concentrations may have also been due to toxicity, which would help to explain the mortality described in this chapter. Studies reporting osteogenic effects of extracts in larvae are rare and mostly limited to compounds which help to promote or preserve bone formation, such as those previously discussed (Chen et al. 2014; Luo et al. 2016). However, there are examples of those which hamper bone formation, such as Dorsomorphin – a small-molecular inhibitor of BMP which reduces zebrafish bone mineralisation (Yu et al. 2008).

Interestingly, *C. spongiosus* (the third and final negative control extract) had very similar *in vitro* activity to *S. muticum*, including increased ALP activity at lower concentrations and concerns over toxicity at higher levels. However, these results do not correlate with the increase in operculum area seen upon treatment with high concentrations of water and saline dissolved *C. spongiosus* extract. This is the first time where *in vitro* data has not correlated to some degree with *in vivo* findings, indicating that hBMSC effects are not always predictive of whole organism effects. The permeability of larval zebrafish to small molecules makes waterborne exposure an easy and reliable method of extract exposure (Wilkinson & Pritchard 2015). However, applying treatments directly to cells, as opposed to systemic exposure to the body of the host, are two very different dosing regimens. *In vivo*, compounds are subject to metabolic processes, directed by the liver, which can affect both the bioavailability of the drug and the structure, leading to the formation of secondary metabolites. These secondary metabolites may be bioactive themselves but can also be toxic. Furthermore, there is often inter-specimen variability, particularly in humans (due to variables such as gender and race), making the pharmakinetics of a compound an important consideration (Rostami-Hodjegan & Tucker 2007) and the reason why *in vivo* testing is crucial.

Finally, *P. squamatus* was again similar in activity to *Osmundea sp.*, and only one concentration (790 µg/ml water extract) significantly increased operculum area. For saline extract treatments, the calcitriol positive control also failed to significantly increase operculum area, which may indicate fish used within this experiment were not responsive to pro-osteogenic treatments. Alternatively, and more likely, was that calcitriol had become degraded upon repeat exposure to light; as after this experiment higher calcitriol concentrations were used, which resulted in expected operculum area increases. *P. squamatus*, like *C. spongiosus*, also had variation between *in vivo* and *in vitro* datasets, though this time it was cell activity that was greater than that of the operculum system. For example, hBMSC differentiation and mineralisation was greatly increased upon exposure to saline *P. squamatus* extract. Other extracts which had similar *in vitro* effects, such as those of *B. fruticulosa* and *C. pallidum*, also displayed notable increases in operculum area. *P. squamatus's* limited operculum system response therefore indicates that bioactivity of this extract is lost *in vivo*, perhaps due to changes in bioactive(s) structure during exposure or whilst being metabolised by larvae.

Whilst high throughput methodologies for detecting osteogenic activity using zebrafish do exist – such as the *in vivo* operculum system (Tarasco et al. 2017) and *ex vivo* scale assay (de Vrieze et al. 2015) – these have not previously been used for large scale screening efforts testing novel compounds/extracts. The closest example of a similar screening effort is that which discovered dorsomorphin (previously described inhibitor of BMP-2), whereby 7500 compounds were tested for their ability to dorsalize zebrafish embryos, i.e. to promote structures at the dorsal pole to the detriment of ventral structures (Yu et al. 2008). *In vitro* screening methods also give some indication the difficulty of this work and the low accompanied success rate in detecting positive activity, i.e. 'hits'. For example one study carried out a high throughput chemical screen of 45,000 small molecules, finding only two series of small molecules with potent osteogenic activity (Han et al. 2009). As such, finding novel *in vivo* activity is also likely to be challenging and therefore this projects results are promising – showing a large proportion of those tested extracts to increase the degree of bone formation in juvenile zebrafish. This is likely to have been influenced by the extraction method used to create powder extracts, as other more conventional extracts showed little *in vitro* activity.

5.4.1.3 Limitations

Based on the original description of this system, its ability to detect pro and anti-osteogenic effects and the results of this chapter, it is a highly useful method of determining *in vivo* osteogenic activity. However, it – along with the work of this chapter – is not without limitations. For example, as mentioned previously, and in the original system description (Tarasco et al. 2017), a more detailed screen of at least 100 different molecules (both novel and previously tested material) would give a better indication of the systems capabilities. Furthermore, as with any biological system, inter-individual variability is a factor and changing the brood pair can have significant repercussions; as shown by the smaller operculum area changes with the brood pair used for later experiments. Within this project, the operculum system – with its shorter running time and excellent repeatability, was used more extensively than the caudal fin regeneration model. Despite this, due to time limitations three tests were planned but not completed, including: testing of water extracts 614 'in-house' and 334 (also 'in-house') and retesting of 615 water extract, to obtain a dataset without the anomalous mortality event described.

Another limitation concerns the comparison of *in vitro* and *in vivo* results, as assays using the same species should ideally be used for both *in vitro* and *in vivo* screening (Kunz et al. 2006). In this study, human derived primary cells were used to identify promising candidates before *in vivo* testing, as opposed to using available fish cell lines such as VSa16 (Tiago et al. 2011). Despite this, *in vitro* data generally predicted and complemented operculum activity very well. Furthermore, as this project concerns finding a bioactive to treat human pathologies, use of hBMSCs gives the greatest likelihood of identifying active compounds at the cellular stage of work. Therefore, mixing results from human derived cells with those of zebrafish is justified, but should still be considered as a potential variable – which may help to explain previously described differences between the *in vitro*/*in vivo* datasets for *C. spongiosus* and *P. squamatus* extracts. Finally, it is also important to note that this system investigates changes in operculum area during its formation, rather than healing in a wound situation. Therefore, whilst informative and indicative of osteogenic potential, the operculum system was also complemented by caudal fin regeneration results, to give a better indication of an extracts healing potential.

5.4.2 Caudal fin regeneration system

5.4.2.1 Caudal fin regeneration system design and benefits

As with the operculum method, the caudal fin regeneration model is a recently described and valuable *in vivo* tool, allowing for the testing/development of existing and novel bioactives able to stimulate skeletal regeneration. Humans have a limited capability to regenerate, whilst other vertebrates have a range of capabilities - such as axolotl (Mexican salamander) which takes approximately 30 days to regrow a limb (Yokoyama 2007). Zebrafish are particularly fast, with this model utilising increased temperatures of 33°C (up from 28°C) to facilitate rapid tissue growth, allowing an entire caudal fin to be re-grown in only 5 days (Boominathan & Ferreira 2012). Fins are comprised from two main elements; lepidotrichia or segmented bony rays and inter-ray mesenchymal tissue - which is covered externally with epidermis. Production of bone matrix and mineralisation of lepidotrichia relies upon osteoblasts, or scleroblasts as they are referred to within the caudal fin. After amputation, scleroblasts are produced from the blastema, a temporary structure of undifferentiated cells with a high proliferative capacity – formed by dedifferentiation of existing cells around the amputation site (Knopf et al. 2011; Cardeira et al. 2016). As seen with the operculum system, the main benefit of this method (other than its speed, low cost and ease of working with zebrafish) compared to those existing within the literature is that it accounts for inter-specimen size variability. This is accomplished through normalising RMA (real mineralised area) measurements by a mean ray width value for each individual, which reduces data dispersion (Cardeira et al. 2016).

Before discussing extract effects, it should be noted that the operculum area and caudal fin regeneration systems measure two very different processes, and therefore test for different extract effects. The operculum is a dermal bone, formed through intramembranous (rather than endochondral) ossification after the development of MSCs into osteoblasts (Hammond & Schulte-Merker 2009). As stated, this is the first dermal bone to ossify in the pharyngeal arches, becoming visible at 3 dpf when embryos have just hatched to produce larvae (Kimmel et al. 2010). Therefore, the operculum system measures *de novo* bone formation of larvae, which would equate most closely to intramembranous bone formation during human foetal development. However, this does not mean that an extract which supports operculum area increases isn't also likely to simulate bone growth in adult humans, as there is likely to be cross over in the mechanisms between the two. Caudal fin regeneration is supported by the dedifferentiation of cells to form a blastema (Knopf et al. 2011), which ultimately produces osteoblasts/scleroblasts to carry out new bone formation. This is most similar to hematoma

and callus formation at sites of human fracture, which help to support subsequent endochondral and intramembranous bone formation (Einhorn 1998). As such, the caudal fin regeneration system allows the ability of extracts to promote tissue regeneration to be tested, which could help to identify a potential treatment to stimulate bone formation after complex fracture or a mobile/soluble additive which could be included during scaffold insertion.

5.4.2.2 Extract effects on caudal fin regeneration

As both water and saline based extracts had shown promise using the operculum system, both were also tested in adult individuals. However, unlike in the operculum model, most powder extracts tested had a limited effect on caudal fin regeneration. For example, neither *P. squamatus* (1358) or *C. spongiosus* (632) water extract had any notable effect on regeneration or osteogenesis. Of these two measures, osteogenesis is most important for this study – as it directly relates to bone formation due to extract treatment. A bioactive able to also stimulate the formation of other tissues, i.e. promoting regeneration, would be an interesting discovery, but is not the focus of this project. Of note, mineralogenic effects can be influenced by changes in regenerative output, leading to false positives; as osteogenesis refers solely to bone formation, whilst regeneration includes the formation of all tissue comprising the caudal fin. As such, it was important to measure regeneration, so that it could be used to normalise bone formation values.

Interestingly, *C. spongiosus* water extract increased the number of high intensity pixels in the regenerated fin, indicating extract treatment increased bone mineral density despite its lack of effect on osteogenesis. One potential explanation for the disparity between these two measures is that pixel intensity analysis is a more sensitive technique, which is better able to detect mild mineralogenic outcomes. This is also supported by results reported in the original study description (Cardeira et al. 2016), which as part of its testing included warfarin treatment; an anticoagulant drug which is known to reduce osteoblast differentiation and alter larval skeletal development (Fernández et al. 2014). Osteogenesis was unaffected upon warfarin treatment (rather than decreasing as expected), though an increase in low intensity and decrease in high intensity pixels was seen – representing the expected reduction in bone density. This appears to indicate that overall sensitivity within this system is lower than that of the operculum method, which may stem from the inherent differences between each growth area. For example, developing larvae have yet to form most skull bones, meaning a

large space is available for growth and bone formation is not constrained by pre-existing structures. Alternatively, whilst adult individuals are completely re-growing fins and therefore technically not constrained, regrowth occurs outwards from existing lepidotrichia to quickly produce a fully functioning fin to mimic that which was lost. Compared to the head area of juveniles, this new fin area is densely packed with tissue, meaning less unoccupied space is available for significant bone formation to occur in. This could therefore explain why bone density, opposed to osteogenesis (which includes size measurements), is a better indicator of pro or anti-osteogenic effects.

The second caudal fin experiment was marked by significant mortality in all but one treatment – that of *C. secundatum* (614) water extract. This could have been due to toxicity of the extracts, though admittedly *C. spongiosus* (632) water extract showed no toxicity when tested previously. One explanation is a difference in extraction efficiency between the two treatments, as each was prepared separately and therefore could have contained differing toxin concentrations. Furthermore, the majority of deaths took place within 24 hours, when a response to the trauma of fin amputation may have contributed to the mortality rate. Therefore, it is difficult to make any conclusions about the toxicity of these extracts. Ideally this experiment would have been repeated but time did not permit.

As with *C. spongiosus* treatment, *C. secundatum* water extract had no significant effect on regeneration or osteogenesis, though did show a different pixel intensity distribution compared to control. Contrary to previous results, pixel analysis indicated *C. secundatum* extract caused a decrease in bone density. This effect doesn't correlate with previous *in vitro* and operculum system results for this extract, as these indicated it was pro-osteogenic and capable of increasing cell growth/differentiation and operculum area. The most likely reason for this disparity is differences in preparation of extracts between the two studies. For example, use of IP injection meant that only 3 µl of extract solution could be injected into an individual, necessitating concentration of extract solutions. As discussed in the methods, this involved further processing steps, including drying and re-solubilisation of extract solutions, which could have impacted bioactive structure and extract composition – particularly for volatile constituents (Abascal et al. 2005). Furthermore, it should be noted that injection of extracts is a very different dosing regimen compared to continuous exposure in water. For example, *C. secundatum* extract may contain a mixture of different bioactives, including one which has a pro-osteogenic effect but is degraded quickly *in vivo*. As such, a continuous exposure, as opposed to a single large dose, may have increased the overall effective treatment level. Comparing to other relevant studies, work involving the regeneration of

caudal fins tends to focus on developmental biology and as such studies reporting extract/compound effects on regeneration are limited. However, one relevant study did undertake a chemical screen for inhibitors of caudal fin regeneration, finding two compounds that inhibited adult regeneration (Oppedal & Goldsmith 2010) – though to a much greater extent that that of *C. secundatum* water extract.

As only one saline dissolved extract was tested in the first two experiments, the final experiment focused on these for many promising red algal species; as well as *C. spongiosus* (632). For this extract, the test concentration was halved, which successfully reduced mortality. Despite this, no significant effect was seen for any measure tested, though pixel intensity analysis did indicate a non-significant reduction in bone density. *P. lyngbyanum* (333) saline extract was almost identical to *C. spongiosus* in its effect, again indicating (though not to a significant degree) a reduction in bone density. As with *C. secundatum* (614) water extract, this result is at odds with previous *in vitro* and *in vivo* results, which indicated a pro-osteogenic extract effect. This disparity could again be feature of extract preparation and exposure method used for these experiments. Interestingly, *C. secundatum* saline extract had the opposite pixel intensity effect to that of *P. lyngbyanum*, *C. spongiosus* and its own water extract, indicating an increase in bone density; though this effect was also not significant. Therefore, retesting with a higher extract concentration may better stimulate bone density increases within regenerating fins. Furthermore, higher extract concentrations may have been beneficial in stimulating an *in vivo* response for all treatments, as only one extract showed significant deviation from control and no mortality was observed. In addition to this, results of this experiment support those of the previous two, showing pixel intensity analysis to be a more sensitive measure than regeneration and osteogenesis. One anomaly that should be discussed is *C. spongiosus*, as water extract showed a small increase (non-significant) in bone mineral density but significant mortality upon retesting, whilst as discussed saline extract had no evident effect. The precise reason for this lack of repeatability is unclear, but the morality may have been due to the separate treatment solutions used between tests of water extracts, whilst alkaline extraction (saline extract) may have had a different composition to that of water.

As in the operculum system, *C. pallidum* (615) extract again caused the most evident response in treatment individuals. Admittedly, adult fish did not respond as obviously to *C. pallidum* treatment as larvae, though unlike in larvae no toxic effect was seen (n.b. only one concentration in adults, rather than a range, was tested). Furthermore, no effects were statistically significant, though this may have been influence by the statistical test used, as

results did not have equal variance and were thus analysed using the non-parametric Kruskal-Wallis test. Despite this, measurements of pixel intensity indicated brighter pixels and therefore a stronger staining in bony rays regenerated in fish exposed to this red algae extract. This indicates that, relative to control, *C. pallidum* extract treatment was promoting the bone density of the regenerated rays, correlating with the previous *in vitro* and *in vivo* operculum effects seen. The slight increase in osteogenesis and decrease in regeneration supports this effect, though these were also not significant in size. The original description of the caudal fin regeneration system only tested warfarin and retinoic acid treatments, neither of which significantly promoted measures of regeneration or mineralogenic activity (Cardeira et al. 2016). As such, comparison of *C. pallidum* extracts effect is difficult and further work is required to better test the ability of this model to detect anti and pro-osteogenic activity.

5.4.2.3 System limitations/future work

The biggest limitation to using this system was that extracts had to be injected into individuals, as too much extract material would have been required for waterborne exposure in large (900 ml) containers. This meant extracts had to be concentrated via drying and re-solubilisation, which as mentioned may have impacted upon bioactive(s) structure. Concentrations were chosen to reflect doses used in in vitro and in the operculum testing. However, injection would have given a larger immediate dose of extract treatments, which could also explain mortality if toxic compounds were present in extract mixtures. Despite this large initial dose, overall treatment concentrations were probably lower than that of the operculum system, as adults were not constantly exposed to extracts; meaning osteogenic effects could have become reduced or lost over time.

Another issue was that the number of samples per group was smaller in this model which meant that statistical power of the experiments to detect an effect was correspondingly lower. This smaller number of repeats was due to limitations involved in using adult individuals. For example, experiments needed to be feasible, so that amputation and treatment of fish could be accomplished within a reasonable time frame, therefore reducing fish stress. Furthermore, ideally only 5-7 adults should be housed in one 900 ml container, whilst the water bath used only comfortably held 6 containers. As such, experiments were limited to testing 6 treatment groups, each containing approximately 5 fish. Future work could focus on addressing these methodological concerns. For example, larger buckets could be used, which would allow more individuals to be included per treatment group and would

thus improve power and repeatability of results. In terms of exposure, waterborne exposure could be used for extracts with the greatest available stocks of material. Alternatively, a second IP injection could be performed partway through the experimental period, to boost circulating extract concentrations; though this could also increase extract and injection related mortality. Another potential method to increase sensitivity of the system would be to try different time points of sacrifice, rather than the 5 days originally described and used so far with this system (Cardeira et al. 2016). In particular, use of a shorter exposure period, perhaps of three days, would likely catch fins in the middle of the regeneration process – allowing differences between control and treatments to be highlighted more easily. Furthermore, this system would also benefit from inclusion of a positive control, such as calcitriol used with larval zebrafish, to confirm its ability to detect anabolic effects between experiments.

As operculum testing gave more interesting results and was faster (3 days compared to 5) to complete, more extracts were tested in it compared to the caudal fin regeneration system. This, coupled with time limitations, meant that testing of some extracts could not be completed – such as water extract of *C. pallidum* and retesting of saline extract at a higher concentration. Despite this, considering the 4-month period available for work, *in vivo* testing detailed within this chapter is a useful addition – giving a better indication of extract activity.

5.5 Summary

This chapter detailed the potential of both water and saline dissolved powder extracts *in vivo*, focusing on their operculum area effects in larvae, but also detailed changes to caudal fin regeneration in adults. Numerous extracts, particularly those from red algae, caused large increases to operculum area during the experimental period, many of which were dose-dependent. This indicated the presence of osteogenic molecules/compounds within these extracts, which were able to stimulate bone formation. Furthermore, for the most part these effects also correlated well with previous measures of *in vitro* activity, giving an indication of how each extract was stimulating osteoblast activity within larvae. Alternatively, with some notable exceptions, such as *C. pallidum* saline extract, most treatments showed a limited potential to stimulate mineralogenic activity and regeneration of adult caudal fins. This is likely due to differences between extract preparation and exposure method between the two systems. However, pixel intensity analysis was a more sensitive measure, which showed

that some extracts treatments were able to both increase and decrease bone density in regenerated lepidotrichia.

Overall, powder extracts tested within this chapter showed promising *in vivo* potential, indicating that these effects may be maintained upon further testing in higher mammals/humans. Future *in vivo* work should focus on further optimisation of the caudal fin regeneration system as detailed, so that more reliable results can be obtained.

Chapter 6

Further discussion, conclusions and future work

6.1 Further discussion

6.1.1 Short summary

The overall aim of this research was to discover a novel marine-organism derived osteogenic bioactive(s). It was envisaged that this would be a potential treatment option to stimulate new bone formation in those suffering from musculoskeletal conditions, such as osteoporosis or healing after complex fracture. The work described in this thesis consisted of three main parts; 1. a large screen for osteogenic activity from a total of 101 marine-organism derived extracts/fractions, mainly using the hFOB cell line (chapter 3), 2. a more detailed screen of powder extracts using hBMSCs (chapter 4) and 3. use of two zebrafish systems to assess osteogenic activity of powder extracts *in vivo* (chapter 5). Screening work was conducted on many extracts from a wide range of taxonomic groups, that were available for testing through a partnership with the Marine Institute Ireland. Whilst mineralising species were hypothesized to contain compounds/molecules that may also promote mineralisation in human cells, the potential effect of other taxonomic groups was largely unknown; therefore no taxonomic group was excluded.

Interestingly, mineralising species, as well as extracts produced with the use of conventional solvents (i.e. material extracted with DCM/methanol), only had a limited effect on osteoblast activity. Instead, powder extracts – the material left over from DCM/methanol extraction which was then dissolved using 0.1M NaOH (neutralised to form a saline extract) or water – had the greatest osteogenic effect. When tested in detail, using hBMSCs, 13 out of 17 powder extracts had a positive effect on either hBMSC proliferation, differentiation or mineralisation. hBMSCs were chosen for more detailed testing, as they are more representative of cells within the human body and therefore give a better indication of pre-clinical potential. Finally, when tested *in vivo*, all extracts identified during *in vitro* work also promoted an increase in operculum area of juvenile zebrafish. Many of these increases were highly statistically significant, further supporting each extracts osteogenic potential and indicating that further *in vivo* testing (for example in mammalian models) may also yield interesting results. Combining both *in vitro* and *in vivo* results, the following powder extracts showed the most osteogenic potential:

- 4 red algae extracts: *Plocamium lyngbyanum* (333), *Ceramium secundatum* (614), *Ceramium pallidum* (615) and *Boergeseniella fruticulosa* (294).
- 1 brown algae extract: *Punctaria sp.* (621).
- 1 deep sea sponge extract: *Psolus squamatus* (1358).

Unfortunately, the exact species of the *Punctaria sp*. extract remains unknown, though substantial efforts are continuing to identify it (as detailed in the discussion section of chapter 4). Therefore, this discussion will concentrate on the remaining species which have been identified, the likely composition of these extracts and their future potential.

6.1.2 Taxonomic descriptions and related studies

Determining accurate species information for each extract is of utmost importance. This information means fresh sample material can be sourced, allowing experiments to be repeated and further work to be carried out. Furthermore, it also gives an indication of other species that may contain bioactives and the likely constituents of each extract (if chemical analysis has not been completed). Determining the species used to source extracts was a limitation of the present study, as for some extracts these records (from the MI) were not available - like the aforementioned *Punctaria sp.* (621). Fortunately, this was not an issue for the majority of powder extracts which showed potential within this project.

6.1.2.1 Plocamium genus

***P. lyngbyanum* (extract 333)**	
Proliferation: maximum +120%	Differentiation: variable, maximum +100% (8 µg/ml)
Mineralisation: maximum exceeds 200%	Operculum area: maximum +50-60%

Plocamium is a genus of red algae commonly found in shallow water areas along Northeast Atlantic coasts, often growing epiphytically on other larger algae (Cremades et al. 2011). The two species sampled in this work, *P. lyngbyanum* and *P. cartilagineum*, are morphologically similar, though genetically distinct (Cremades et al. 2011). So far very little work has been conducted on this algal species, both in terms of chemical composition and bioactivity. It is known to contain volatile halogenated metabolites (Kladi et al. 2005), whereby halogens present in seawater are incorporated into organic compounds with between 1 and 30 carbon atoms. However, these compounds are thought to largely aid defence against microorganisms and are unlikely to be osteogenic. In fact, some studies indicate they may be cytotoxic and instead show greater antibacterial or antioxidant potential (Smit 2004; Kladi et al. 2005).

One relevant study focused on chemical composition and showed that, in addition to halogenates, other volatiles including alcohols, esters and aldehydes, as well as four organic acids, were present in *P. cartilagineum* (Valentão et al. 2010). Whilst proteins and

polysaccharides are the most common osteogenic organics present in algae, those found in this study may also show activity. Also of note, Valentão *et al.* showed considerable antioxidant activity of *P. cartilagineum*, whereby aqueous extracts were tested for their scavenging potential of free radicals and reactive oxygen species. Similar capabilities were also observed in extracts from three species of Romanian algae, including the genus *Ceramium* (Horincar et al. 2011). Antioxidants are known to support osteoblast action, as even low exposure to molecules such as H_2O_2 can otherwise reduce mineralisation and gene expression of osteogenic markers (Arai et al. 2007). In fact, many osteogenic extracts possess antioxidant capabilities, such as nacre water soluble matrix (Chaturvedi et al. 2013) and sulphated polysaccharides of algae like *Laminaria japonica* (Wang et al. 2008), including the well characterised fucoidan (Mak et al. 2013). *In vivo*, this could explain *Plocamium sp.* extracts ability to support larval operculum growth, though it is not the cause of the increased *in vitro* activity described. Also, it seems unlikely that antioxidant capabilities would be solely responsible for the positive effects during the short 3-day exposure period used with the operculum system.

6.1.2.2 Ceramium genus

***C. secundatum* (extract 614)**	
Proliferation: maximum +80%	Differentiation: maximum +100%
Mineralisation: no pronounced effect	Operculum area: maximum +50-60%

***C. pallidum* (extract 615)**	
Proliferation: maximum -40%	Differentiation: maximum +150%
Mineralisation: maximum exceeds 200%	Operculum area: maximum +86%

Ceramium is another genus of Rhodophyta, with a ubiquitous distribution across Northeast Atlantic coasts - including multiple species in the British isles (Maggs & Hommersand 1993). Of these, *C. secundatum* and *C. pallidum* are closely related, belonging to different subclades of the *Ceramium* genus (Maggs et al. 2002). Morphologically they are readily distinguishable because *C. pallidum* is regularly branched every 4-8 segments, whereas *C. secundatum* is branched at intervals of 9 or more segments. *Ceramium sp*. are typically smaller than 30 cm in length and are found in both shallow and deep water coastal areas. This genus, like those of all the samples discussed in this chapter, has very little published information available concerning potential bioactive components. What is apparent is that, like those derived from *Plocamium sp.*, extracts may have both antiviral (Serkedjieva 2004) and antibacterial (Dubber & Harder 2008) activities. The current work is the first report of osteogenic potential.

6.1.2.3 Boergeseniella fruticulosa

***B. fruticulosa* (extract 294)**	
Proliferation: variable small +/- changes	Differentiation: maximum +150%
Mineralisation: maximum exceeds 200%	Operculum area: maximum +40-50%

B. fruticulosa is a small (up to 15 cm) branched red algae, which lives epiphytically on other algae or rocks. This species has a wide distribution, including eastern Atlantic habitats such as Morocco, the Mediterranean, Britain and also Ireland (Rindi & Guiry 2004). As with *Plocamium* and *Ceramium*, most research conducted to date on extracts from *B. fruticulosa* have been focused on antiviral and antibacterial activity, although even these studies are limited. For example, one paper investigated whole extracts from 23 red algae species, including *B. fruticulosa*, *P. cartilagineum* and *C. virgatum*, for their antiprotozoal, antimycobacterial and cytotoxic effects (Allmendinger et al. 2010). All extracts showed activity in at least one measure, such as against the parasite *T. brucei rhodesiense* – though *C. virgatum* was the most potent. As in the current study, no chemical analysis was completed as the work focused on screening and identifying activity, though secondary metabolites were identified as the likeliest source of activity; such as alkaloids, polyphenols, sulphated sugars, sterols and halogenated mono and diterpenes.

6.1.2.4 Rhodophyta

As most active extracts described in this chapter come from Rhodophyta species it suggests the bioactive component might be common to members of this phyla. Overall, red algae are known to have a particularly high protein content, of up to 47% of their dry matter, along with high levels of polysaccharides and minerals (Cian et al. 2015). For polysaccharides, an obvious comparator is fucoidan, which shows similar activity profiles from many different species of brown algae. It is particularly relevant to this work, as low treatment concentrations (like those used with *P. lyngbyanum* extract) are able to significantly increase MSC proliferation, (Kim et al. 2015). However, red algae do not contain fucoidan, though they do contain cell wall associated sulphated polysaccharides such as carrageenans and agar (Usov 1998; Wijesekara et al. 2011). Whilst these polysaccharides have been incorporated in scaffolds which themselves have osteogenic potential (Li et al. 2015), there are no reports of activity when they are included as mobile/soluble additives. In terms of minerals, those derived from red algae have well-known and characterised mineralogenic effects, such as the marine derived multi-mineral Aquamin. This commercial extract is able to promote *in vitro* mineralisation (Gorman et al. 2012) and has even been tested in basic and underpowered *in*

vivo human trials (Frestedt et al. 2008; Frestedt et al. 2009). However, the present study used only non-mineralising species and these are therefore unlikely to contain biologically relevant mineral levels. This leaves proteinaceous material as the most likely active component of each extract, though if true the extraction method and solvent choice will have likely impacted protein structure (see section 6.1.3).

Overall, studies reporting red algae based bioactives specific to bone tissue are very limited. One notable exception to this is a red algal based compound called floridoside, which is a glycerol glycoside metabolite of *Laurencia undulata* (Ryu et al. 2015). Floridoside was shown to promote differentiation, increased ALP levels, mineralisation and expression of factors including type 1 collagen, Runx-2 and Osterix (Ryu et al. 2015). Before this study, floridoside had not been investigated for its highly promising osteogenic potential, thus highlighting the importance of testing novel extracts. Furthermore, this compound is also produced by other species of red algae such as *Galdieria sulphuraria* (Martinez-Garcia & van der Maarel 2016) and *Mastocarpus stellatus* (Courtois et al. 2008), and may therefore also be present in the red algae described in this chapter. However, chemical analysis is needed to determine this.

6.1.2.5 Psolus squamatus

***P. squamatus* (extract 1358)**	
Proliferation: maximum -50%	Differentiation: maximum +150%
Mineralisation: maximum exceeds 200%	Operculum area: maximum +28%

P. squamatus is a species of deep sea sponge which is typically found at more than 200 m depth along continental slopes (found at approximately 2000 m in this study), but may also appear attached to stones in shallower water areas. This species, like the rest of those in this chapter, has a fairly ubiquitous distribution, found in waters off Mexico, Norway, British Columbia and the British Isles (Southward et al. 2006). The vast majority of published research mentioning *P. squamatus* is concerned with describing its distribution in different areas, whilst to date there have been no published reports of bioactives or chemical analysis of extracts from this species. As such, no comparisons can be made to the activity described in this thesis and speculation on active components is limited. Normally, deep sea sampling is limited by the difficulty and cost associated with collecting more sample material (Van Dover 2011), or due to issues of conservation (Davies et al. 2007). However, as mentioned *P. squamatus* is a fairly common species with a ubiquitous distribution in both shallow and deep-water habitats. Therefore, sourcing fresh sample material should be easier than with other deep-water species. Furthermore, use of chemical synthesis would mean that once

bioactivity has been associated with a molecule or compound it could then be reproduced (depending on its complexity), making further sampling unnecessary and addressing concerns about species depletion. Therefore, *P. squamatus* remains an interesting extract able to stimulate hBMSC differentiation, mineralisation and operculum growth, which warrants further study.

6.1.3 Considerations for bioactive composition

The main limitation of this project is that the specific active component(s) for each extract has not been identified. When screening such large numbers of extracts, chemical analysis is not feasible –and often unnecessary, as many do not display significant osteogenic activity. Therefore, chemical analysis of the promising bioactive powder extracts will be the focus of future work. This is detailed further in section 6.3, but will involve determining overall extract composition, fractionation, repeat bioactivity screening and then eventual characterisation of each compound.

Although beyond the original scope of this research project, some preliminary chemical analysis has been conducted and includes Gas Chromatography – Flame Ionisation Detector (GC-FID) to test for fatty acids, which was completed for all samples mentioned in this chapter (along with others, see appendix 9 and 11) and mass spectrometry analysis for extracts from Irish samples of *P. cartilagineum*, UK and Irish samples of *C. secundatum* and *C. pallidum* (see appendix 10 and 12). Fatty acid analysis showed no obvious positive results, with only relatively weak peaks seen for all powder extracts. Furthermore, these peaks did not correlate with any FAME standards, suggesting they were either impurities, or that fatty acids present were at a concentration below the detection limit of the system. Mass spectrometry data were more interesting, showing a large number of peaks for each extract tested. Taking extract 615 from *C. pallidum* (009) as an example, approximately 17 peaks were evident (as shown in appendix 12). This indicates that powder extracts are a complex mix, containing a number of components which may contribute to the overall activity described in chapters 4 and 5.

Initial treatment of samples at the Marine Institute included the use of dichloromethane and methanol. Powder extracts were left over from these extractions and therefore anything removed by these solvents will not have been present in the final extract. DCM and methanol are likely to have extracted a wide range of organics from the original sample material, as DCM - with its intermediate polarity - is a good general-purpose solvent for mid to low

polarity organics, whilst methanol is a polar protic solvent (Lagowski 1978). Their pairing was likely used to dissolve as much organic material as possible, as anything missed by DCM may have dissolved in methanol – such as remaining polar organic compounds. Of the material left over as a powder extract for dissolution at QUB, use of water and 0.1 M NaOH solvents will have probably removed different organic material. Water, with its high polarity, is an efficient solvent for organics such as alcohols, some amines, acids, esters, ketones and aldehydes (Marcus 1998). As such, the active component(s) extracted with water, as for *Punctaria sp.*, is difficult to determine. Alternatively, NaOH is best known for its ability to dissolve carboxylic acids and phenols, through the formation of polar carboxylate or phenoxide groups. For algae, this again supports the idea of the bioactive component being proteinaceous, whilst phenols are also a common constituent of extracts from these eukaryotes. Furthermore, previous studies have reported that extracts from algae which contain high phenol levels are able to promote osteogenic activities both *in vitro* (Surget et al. 2017) and *in vivo* (Park et al. 2012).

Another important consideration is the effect of solvent choice on the chemical structure of organic molecules/compounds. Of particular importance to this work is the impact of alkaline treatment, as even at a 0.1 M concentration NaOH is likely to have influenced the structure of different components within each extract, particularly proteins and polysaccharides. Proteins are most likely to have been denatured, except perhaps more robust glycoproteins, creating smaller proteinaceous molecules – whilst polysaccharides will probably have been affected similarly. One relevant study extracted polysaccharides from the red algae *Gracilaria corticata*, finding that alkali pre-treatment reduced the yield and molecular weight of cold water extracted polymers; due to degradation of the polysaccharide backbone (Mazumder et al. 2002). Despite these concerns, use of alkali extraction was still deemed to be a useful extraction method for this study. This was based on other studies' use of 0.1 M NaOH - which found it to be an effective extraction solvent, particularly for ensuring a high protein yield (Shen et al. 2008; P. A. Harnedy & FitzGerald 2013b; P. a. Harnedy & FitzGerald 2013). Finally, it is important to note that molecular content changes greatly based on the extraction method used. Therefore, the test solutions detailed in this work are likely to be very different from those of other studies, such as that of Valentão et al. (2010); which used an aqueous rather than alkaline extraction to produce extracts from lyophilized *P. cartilagineum* material – meaning extracted components of the two may be very different and inferences on consitutents are hard to make.

6.1.4 Future potential

Of those marine derived osteogenic bioactives described to date, fucoidan and nacre show good pre-clinical potential. Approximate maximum/minimum *in vitro* (proliferation, differentiation and mineralisation) and *in vivo* effects of both extracts have been summarised in table 6.1, along with the same measures for powder extracts described in this chapter. Such summation is difficult, considering the variability between studies in terms of cell type, seeding densities, other experimental conditions, types of each extract and *in vivo* models used, to name but a few variables. Despite this, noting the approximate maximum or minimum percentage change gives a good indication of fucoidan, nacre and powder extracts general effects.

Overall, powder extracts compare favourably with both fucoidan and nacre, arguably outperforming them in some measures of activity. Proliferative effects of the 5 powder extracts are the most variable, ranging from approximate effects on cell number of -50 to +120%. Comparatively, only one nacre related study reported a greater effect, showing MC3T3-E1 cell viability to be increased 150% upon treatment with *P. fucata* mantle gene 3 (Wang et al. 2011b); though other studies testing water soluble nacre fractions reported no significant increases of cell viability or proliferation. Comparatively, fucoidan performed more consistently, increasing cell viability/proliferation in the range of 20-80%. Measures of cell differentiation and mineralisation were more comparable between the three groups whereby, ALP activity was increased in all studies, ranging from 100-150% for all powders tested, between 20 and >200% for nacre-based extracts and between 30 and >200% for fucoidan. As with ALP activity, mineralisation levels were increased in all studies, with powder extracts and nacre showing the greatest overall increases – which often exceeded 200%, whilst fucoidan ranged between 40 and 190%.

In vivo activity is even more difficult to compare between studies than *in vitro* work, as those models used vary greatly in their reported measures. Considering its *in vitro* potential there are a surprising lack of studies testing fucoidan *in vivo*, with most work testing scaffolds incorporated with fucoidan for their *in vitro* osteogenicity (Puvaneswary et al. 2016; Venkatesan et al. 2014; S Igondjo Tchen Changotade et al. 2008). One study did test the sulphated polysaccharide in a bone-growth mouse model, but found no bone formation after 5 or 8 weeks (Pereira et al. 2014). Comparatively, nacre has been tested more comprehensively and is known to fuse directly with bone when implanted in both rats (Liao et al. 2002) and sheep (Atlan 1999; Berland et al. 2005), whilst one trial was even conducted in humans (Atlan et al. 1997; Westbroek & Marin 1998). Contrary to the conventional route

of bioactive discovery, this trial was undertaken before significant *in vitro* and animal model testing and showed that nacre was able to stimulate healing in bone maxillary defects of 8 female patients; fuelling interest in nacreous and other marine-organism derived extracts. Comparing either fucoidan or nacres *in vivo* potential to that of powder extracts tested in zebrafish is difficult. However, powder extracts showed an excellent ability to increase the operculum area of juvenile fish. For example, *C. pallidum* extract has shown the greatest promotion of operculum area of those molecules/compounds/extracts tested in the model system to date. This may indicate that upon further *in vivo* testing, as with nacre, powders could stimulate bone growth in higher mammals.

Table 6.1: summary of the effect of fucoidan, nacre or powder extracts on *in vitro* proliferation, differentiation and mineralisation of osteoblast relevant cells, as well as *in vivo* measures. The approximate maximum percentage increase or decrease reported for each measure is shown, along with the citation for each study. Green indicates an increase in activity and red a decrease. Fucoidan images from seabioresources.com, nacre from spey.com.

	 Fucoidan	 Nacre	 Powders
In vitro proliferation/ viability	- *Hwang et al. 2016:* +50% cell viability (2 mg/ml) - *Cho et al. 2009:* +20% cell viability (10 µg/ml) - *Kim et al. 2015:* +80% proliferation (10 µg/ml)	- *Chaturvedi et al. 2016:* slight decrease cell viability (0.5%) - *Wang et al. 2011:*+150% cell viability (*P. fucata* mantle gene 3) - *Moutahir-belqasmi et al. 2001:*no effect(water soluble fraction)	- *P. lyngbyanum:* +120% - *C. secundatum:*+80% - *C. pallidum:*-40% - *B. fruticulosa:* variable +/- - *P. squamatus:*-50%
In vitro differentiation (ALP activity)	- *Hwang et al. 2016:* +30% (2 mg/ml) - *Cho et al. 2009:* +70% (10 µg/ml) - *Kim et al. 2015:* >200% (1 µg/ml) - Pereira et al. 2014: +70% (10 µg/ml)	- *Chaturvedi et al. 2016:* >200%(0.1%) - *Kim et al. 2012:*+60% (water soluble fraction) - *Wang et al. 2011:*+100% (*P. fucata* mantle gene 3) - *Moutahir-belqasmi et al. 2001:*+20% (water soluble fraction)	- *P. lyngbyanum:* variable +100% - *C. secundatum:*+100% - *C. pallidum:*+150% - *B. fruticulosa:*+150% - *P. squamatus:*+150%
In vitro mineralisation	- *Hwang et al. 2016:* +40% (2 mg/ml) - *Cho et al. 2009:* +70% (250 µg/ml) - *Kim et al. 2015:* +190% (1 µg/ml) - Pereira et al. 2014: +70% (10 µg/ml)	- *Chaturvedi et al. 2016:* >200%(0.025%) - *Kim et al. 2012:*+150% (water soluble fraction) - *Wang et al. 2011:*increase qualitative (*P. fucata* mantle gene 3) - *Brion et al. 2015:*>200% (100 µg/ml)	- *P. lyngbyanum:*>200% - *C. secundatum:* no pronounced effect - *C. pallidum:* >200% - *B. fruticulosa:* >200% - *P. squamatus:* >200%
In vivo potential	- Pereira et al. 2014: no effect bone growth mouse model. - Other studies limited.	- Nacreous impants fuse with rat (Liao et al. 2002) and sheep bone (Atlan et al. 1999, Berland et al. 2005) - maintained long term.	- *P. lyngbyanum:*+50-60% - *C. secundatum:* +50-60% - *C. pallidum:* +86% - *B. fruticulosa:* +40-50% - *P. squamatus:* +28%

6.2 Conclusions

Due to the incidence and cost associated with treating musculoskeletal conditions there has been a drive to find novel treatment options from natural sources, as in other areas of medicine. This work sought to address this need, by screening marine-organism derived extracts for their osteogenic potential, specifically to find an anabolic bioactive that might one day be used to promote bone formation in patients; such as those suffering from osteoporosis. Other studies have conducted similar work, but due to the sheer number and variety of marine species research in this area remains in its infancy; meaning discovery potential is high. Furthermore, published studies do not always characterise activity fully – for example by leaving out the response of primary cells or not testing extracts/compounds *in vivo*. Whilst impractical to include all measures of *in vitro* and *in vivo* activity, this study adopted a comprehensive approach, investigating; cytotoxicity, proliferation, differentiation and mineralisation *in vitro* (using a hFOB cell line and primary hBMSCs), as well as bone formation and regeneration *in vivo* (using zebrafish).

Throughout this work over 100 extracts were screened, with the majority showing little to no promising activity. However, powder extracts were identified as a potential source of bioactives towards the end of the screening process. Subsequent work demonstrated an effective extraction method and the ability of powder extracts to stimulate the activity of hBMSCs. This osteogenic potential was retained *in vivo* upon testing using larval zebrafish, with many extracts stimulating increases in operculum area. Main conclusions for each body of work are summarised as follows;

Method development — **Chapter 2**

- hFOB use was justified for screening work and an effective seeding density of 1 x 10^4 was chosen.
- Limitations with PicoGreen assay as a measure of cell proliferation were described and crystal violet assay was chosen as replacement.
- Effective use of crystal violet assay was developed and described.
- Solvent toxicity: Tween20 was toxic at all concentrations, above 0.1% DMSO caused dose-dependent decreases in cell proliferation, ethanol was less toxic - though concentrations exceeding 0.5% reduced some measures of cell activity.
- 0.1% DMSO and 0.5 or 1% ethanol were chosen for future work.
- hBMSCs were chosen to better describe *in vitro* bioactivity, ethical approval was granted for their use and cells were derived from 8 donor tissue samples. A 2 x 10^4 seeding density was chosen for future work using these primary cells.

- Use of PBS during alkaline phosphatase activity was shown to stop detection of ALP activity, likely due to binding of phosphate within the buffer to the active site of the enzyme.
- Various solvent concentrations were tested in the ALP assay, to determine effects on activity for both hFOBs and hBMSCs. hBMSCs were shown to have the best ALP expression overall.
- hFOB ability to mineralise was again lower than that of hBMSCs and therefore hBSMCs were chosen for future mineralisation work.
- Various media recipes were tested using the mineralisation assay, with 50 µg/ml A-2-P, 10 mM β-glycerophosphate and 0.01 µM dexamethasone the most effective for hBMSCs.

Screening for osteogenic activity **Chapter 3**

Overall: a total of 101 marine-organism derived extracts were tested for their osteogenic activity in this project. Many of these extracts were repeat tested, often over a range of different concentrations and sometimes using more than one solvent.

Batch of 43 extracts dissolved in DMSO (0.1% concentration):

- DMSO dissolved extracts were non-toxic, whilst many decreased hFOB cell death levels (day 1).
- In general, DMSO extracts only caused small responses in terms of hFOB metabolic activity (day 1), proliferation (day 4, 7) and hBMSC differentiation (day 7). Some significant effects were observed, but these were not large enough to warrant further investigation.

Batch of 25 extracts dissolved in ethanol (0.5% concentration):

- Extract 324 was toxic, but remaining extracts were similar in value to control – with only small significant increases/decreases in hFOB cell death seen (day 1).
- No ethanol dissolved extracts notably increased hFOB proliferation, though several caused large decreases (day 1). Similarly, of those extracts tested, none caused a significant increase in hBMSC differentiation (day 7), though several large and significant decreases in activity were seen.

Powder extracts:

- No toxicity data was presented, as fine particulate matter in powder extracts affected results – due to the sensitivity of the LDH assay.
- hFOB proliferation data (day 1, 4, 7) was presented for 10 powder extracts, many of which caused significant increases in cell number.

Powder extract potential — **Chapter 4**

Overall: a total of 17 powder extracts were tested (for at least one activity measure) in this section, using hBMSCs. Results were presented for cell proliferation, differentiation and mineralisation.

Proliferation:

- Of those extracts which increased proliferation, those from *P. lyngbyanum* (333), *P. cartilagineum* (334), *C. secundatum* (614 'in-house') and *Punctaria sp.*(621) were most significant.
- Remaining extracts either had a limited effect on proliferation or decreased it. Of those that caused decreases, extracts from *S. muticum* (840) and *C. spongiosus* (632) were most significant.

Differentiation:

- The majority of extracts caused at least a small increase in ALP activity at one concentration, with lower concentrations being particularly active.
- Large significant increases were seen with extracts from *C. secundatum* (614), *Punctaria sp.*(621), *C. spongiosus* (632), *C. pallidum* (615), *B. fruticulosa* (294) and *P. squamatus* (1358).

Mineralisation:

- Most extracts caused a promotion of hBMSC mineralisation, especially at higher concentrations and later timepoints of day 21.
- Exceptions to this were *C. secundatum* (614), *C. spongiosus* (632) and *S. muticum* (840), which had no significant effect on mineralisation levels.
- 'In-house' extractions of *P. cartilagineum* (334) and *C. secundatum* (614) caused the greatest consistent increase in mineralisation level of those extracts tested.

In vivo bone growth and regeneration — **Chapter 5**

Overall: a total of 11 powder extracts, dissolved in either water or saline (0.1 M NaOH extraction) solution, were tested using an operculum bone growth or caudal fin regeneration system.

Operculum bone growth:

- A preliminary test confirmed that saline solution was not toxic to zebrafish larvae at up to 10% concentration.
- Most powder extracts caused an increase of operculum area, many of which were large. Higher extract concentrations had the best effect, often exceeding growth seen with the calcitriol positive control.

- Both water and saline dissolved extracts showed potential, though activity sometimes differed between the two for extracts from the same species – indicating different bioactives were present in each.
- Extracts from *Osmundea sp.* (625), *S. muticum* (840, saline) and *P. squamatus* (1358, saline) showed limited activity.

Caudal fin regeneration:

- 12 extracts were tested in the caudal fin regeneration system.
- Of those extracts tested, there were no significant effects on the degree of regeneration or osteogenesis in regenerated caudal fins.
- Pixel intensity analysis was the only measure to show significant extract effects. *C. pallidum* (615, saline) extract caused a significant reduction in low intensity and promotion of high intensity pixels – indicating increased bone mineral density. *C. secundatum* (614, water) extract had the opposite effect, indicating reduced bone mineral density.
- The caudal fin regeneration system was thought to be less sensitive than the operculum system, whilst the use of IP injection meant the effective concentration over the growth period was also reduced for adults compared to juveniles.

The work presented in this thesis represents the first stage of drug discovery, providing the first reported evidence of osteogenic activity in extracts from a range of marine organisms, specifically four species of red algae, one species of brown algae and a species of deep sea sponge. Further work is now needed to elucidate the bioactive component of each extract and understand the mechanisms of *in vitro* and *in vivo* activity. Taking *Plocamium* as an example, extracts from this genus promoted a 2-fold proliferation and an 8-fold mineralisation increase, compared to control, with as little as 7 µg/ml treatment concentration; whilst *in vivo* operculum growth response was also excellent. Such potent activity indicates that this extract, along with others, contains bioactives that may be useful in stimulating bone growth in humans. Though this has yet to be confirmed through trials, if true such a treatment would likely help to promote bone growth in those suffering from conditions such as osteoporosis; allowing increases in bone mineral density, a better quality of life and reduced costs for NHS services.

6.3 Future work

Directly related to this project:

- Chemical analysis: each extract should be tested again via mass spectrometry (including negative ionization), using more concentrated solutions to gain a better idea of how complex each extract is. Extracts should then be fractionated, using a technique such as preparative high performance liquid chromatography (HPLC), allowing retesting of each fraction to confirm osteogenic activity. Compounds in each active fraction could then be identified, i.e. using mass spectrometry.
- More extensive re-extraction from fresh algal samples is required to ensure reproducibility.
- After chemical analysis has been completed the mechanisms of action for osteogenic activity should be investigated, including gene expression analysis for osteocalcin, osteopontin, bone sialoprotein and Cbfa-1/Runx2.
- Further *in vivo* testing could include use of several zebrafish based systems/assays to further understanding of extract effects *in vivo*. For example, mutant/transgenic lines are available which express pathologies similar to human musculoskeletal conditions, such as osteoporosis and osteogenesis imperfecta (Laizé et al. 2014). These lines could be used to directly test if extracts are able to alleviate or rescue these pathologies. Furthermore, transgenic lines are a powerful reporter of osteogenic activity, such as those lines bred to express fluorescent markers like green fluorescent protein (GFP) and mCherry at sites of bone formation (Knopf et al. 2011). These could give a visual and quantifiable measure of different aspects of osteoblast development stimulated by each extract, such as expression of key markers like Runx2 and osteocalcin. Finally, one especially interesting method is an *ex-vivo* model using zebrafish scales from sp7:luciferase transgenic adults, where SP7 (osterix) was used as an approximation of osteoblast activity (de Vrieze et al. 2015). This model showed good conservation of activity from known bioactives and also allowed discovery of novel compounds. Furthermore, fish only needed to be anesthetised to remove scales rather than killed, whilst scales were easily cultured over a 3-day period in 96 well plates – using media to sustain zebrafish osteoblasts. This *ex-vivo* scale based assay therefore represents a good potential option for cheap, rapid and accurate screening of many compounds, which could be utilised in future screening of compounds at QUB (as opposed to using the zebrafish facility in Portugal).

- Further screening based on phylogenetic analysis. The genera *Plocamium* and *Ceramium* both had two species which showed activity, indicating other related species may also contain bioactives. This work should also include a seasonal and geographical component, to see how activity varies based on sample site location; following on from the brief investigation of this with *P. cartilagineum* and *C. secundatum* extracts.
- Algal extracts often have some degree of antioxidant capabilities and therefore powder extracts could be tested for this, for example using the DPPH assay *in vitro* and in zebrafish through exposure to a stressor such as heavy metals or UV radiation.

Recommendations for the field:

- A greater focus on *in vivo* testing for promising extracts, to ensure activity is maintained within a whole organism.
- Greater use of hBMSCs, as these give a better indication of how cells will respond within the human body.
- Investigations into the effect of extracts (such as those of this work and similar studies) on cell senescence. Cell senescence and osteoporosis are both age related and therefore an extract which can reduce senescence, whilst also promoting osteogenicity, could be very clinically relevant.

7

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7. References

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8

Appendix

Appendix 1 – ethical consent form for bone marrow collection

Queen's University Belfast

Belfast Health and Social Care Trust

School of Nursing
and Midwifery
Medical Biology
Centre
97 Lisburn Road
Belfast BT9 7BL
02890 972171

Consent Form

LREC ref no.:

Title of Project: Discovering bioactive compounds for bone growth: the osteogenicity of marine-organism derived extracts.

Name of Lead Investigator: **Dr. Susan Clarke**

	Please Initial Box
1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
3. I agree to donate bone marrow.	☐
4. I agree that my bone marrow can be used in future studies.	☐

Name of Research Subject *(please print)*	Date	Signature
Name of Research Team Member	Date	Signature

Appendix 2 – Patient information sheet

Queen's
University Belfast

School of Nursing
and Midwifery

Medical Biology
Centre

97 Lisburn Road

Belfast BT9 7BL

Patient Information Sheet

Evaluation of Osteogenic Potential of Marine Extracts

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the information below carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not to take part.

About the Study

We are testing extracts of marine creatures to identify new drugs which might have the potential to aid bone healing. An important step in testing these extracts is seeing how they affect cells in the laboratory, such as if they grow and develop better and to ensure the extracts do not damage the cells. The best cells to use for these experiments are cells from human bone marrow which have the potential to become osteoblasts. These cells are responsible for making bone.

We are asking you to donate a sample of bone marrow for these laboratory experiments to test a number of marine extracts we have collected. As this is a common type of research study used in our laboratory, we are also asking for your permission to keep any unused cells for future similar studies. If you do not consent to this, your cells will be safely disposed of when the experiment is completed.

Why have I been chosen?

Patients who require spinal surgery involving the placement of metal screws into their spinal vertebrae (bones) are being asked to participate.

Do I have to take part?

No. It is up to you whether to take part or not. If you decide to participate you will be asked to sign a consent form. You may withdraw from the study at any time by contacting the researcher named at the bottom of this information sheet. You do not have to give a reason. If you decide not to take part, this will not affect the treatment you receive.

What will happen to me if I take part?

If you decide to take part in the study you will be asked to sign a consent form to allow the surgeon to collect a bone marrow sample during your surgery. During the routine placement of screws the bone marrow cavity of the spinal vertebrae (bones) is accessed. If you agree to participate approximately 6-8 mls of bone marrow will be collected through the hole already made for screw insertion. The screw will then be inserted as usual. No further access into the bone marrow cavity will be necessary. Your surgery will then continue as normal.

What is required of me?

If you decide to participate in the study you will be asked to sign a consent form to allow your surgeon to take a bone marrow sample during your surgery. No further participation is required by you for this study. You should follow your consultant's instructions as normal following your surgery.

What are the possible risks of taking part?

There are no additional risks to you above those usually associated with your surgery.

What are the possible benefits of taking part?

Taking part in this study will have no clinical benefit to you and will not affect your treatment. The information we get from this study may help us to treat patients in the future more effectively.

Confidentiality- who will have access to the data?

It will be necessary to obtain general information from your hospital notes. This information will be accessed only by your clinical team and will be fully anonymised before being passed to members of the research team. No information that can identify you such as name, address or hospital no will be available outside the clinical environment.

What will happen to my tissue at the end of the research?

With your consent your tissue may be used for future similar experiments. If you do not consent to this then it will only be used for this experiment and when the testing is completed, your sample will be destroyed.

What will happen to the results of the research?

The results will be published in a scientific journal. You will not be identified by name in any publication.

Who is organising and funding the research?

The research is funded by the School of Nursing and Midwifery, Queen's University Belfast in association with NHS R&D Office and a Beaufort Marine Research Award with the support of the Marine Institute, Ireland.

Who has reviewed the study?

The research has been approved by North East-Tyne and Wear South and by research governance at Queen's University Belfast and Belfast Health and Social Services Trust.

Contact for further information

Research: Dr Susan Clarke PhD 02890 97 2171

You will be given a copy of this information sheet to keep for your records.

Appendix 3 – basic information collected from patient

Age:

Height:

Weight:

BMI:

Gender:

Drugs:

Co-morbid conditions:

Inclusion: age 18-65, able to consent for self

Exclusion: rheumatoid arthritis, osteoporosis, other metabolic bone diseases, or taking bisphosphonates, long term steroid use and statins

Appendix 4 – donor information table

Donor number	Age	BMI	Gender	Drugs	Co-morbid conditions
00 1	20	21.3	Male	Inhaler	Asthma
00 2	51	24	Male	N/A	N/A
00 3	22	24.8	Female	N/A	N/A
00 4	38	23.7	Male	anti-epilepsy	N/A
00 5	21	28.5	Male	N/A	N/A
00 6	38	24.5	Male	N/A	N/A
00 7	29	24.7	Male	N/A	N/A
00 8	39	28.8	Male	N/A	N/A

Table 8.1: containing basic information for each of the 8 bone marrow donors of this study. Included is donor number, age, BMI, gender, drugs taken and any co-morbid conditions. Some information, such as the date of marrow collection, has been excluded – to ensure the anonymity of donors.

Appendix 5 – ALP assay optimisation

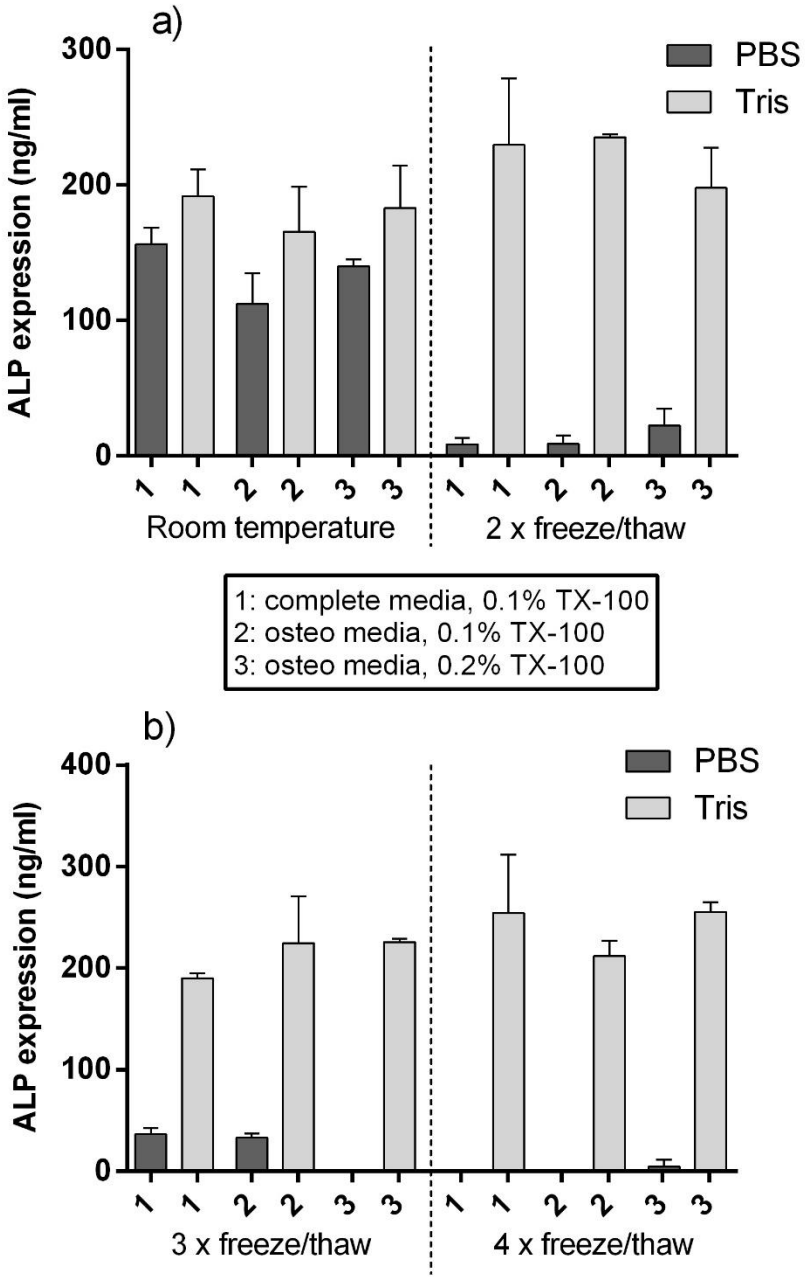

Figure 8.1: results from an alkaline phosphatase assay performed on hFOBs, split for convenience between a) and b). Cells were plated at a $1x10^4$ cells/cm^2 density, given 24 hours to attach and then cultured for 7 days in either complete or osteogenic media. Subsequently, cells were lysed via addition of a PBS or Tris buffer containing 0.1 or 0.2% Triton X-100, and either kept at room temperature or subjected to 2, 3 or 4 cycles of freeze thaw. Results are presented as the mean +/- standard deviation of duplicate values *(n=2 or 3)*, *$p<0.05$ for treatment compared with medium only control.

Appendix 6 – Summary of tested extracts/fractions

Table 8.2: table showing the extracts tested (ID number), their common name and the corresponding genera/species/description of each. A (1) or (2) indicates both extracts were from the same species, but collected at different times/by different individuals.

Extract number	Common name	Genera/Species/description
	DMSO dissolved extracts	
MR-01	Red algae	*Bonnemaisonia hamifera*
MR-02	Coastal sponge	*Porifera demospongiae*
MR-03	Brown algae	*Bifurcaria bifurcata*
MR-04	Coastal sponge	Porifera; Order Poecilosclerida
MR-05(1)	Coastal sea squirt	Chordata; Class Ascidiacea
MR-06	Coastal sponge	*Halichondria panicea*
MR-07	Red algae	*Anotrichium barbatum*
MR-08	Star fish	*Asterias rubens*
MR-09(2)	Coastal sea squirt	Chordata; Class Ascidiacea
MR-10	Coastal sponge	*Haliclona indistincta*
MR-11	Red algae	*Griffithsia corallinoides*
MR-12	Red algae	*Bonnemaisonia hamifera*
MR-13	Coastal sponge	Porifera; Order Poecilosclerida
MR-14	Coastal sponge	Dictyoceratid sponge
MR-15	Green algae	*Ulva linza*
MR-16	Brown algae	*Cystoseira tamariscifolia*
MR-17	Red algae	*Polysiphonia elongata*
MR-18	Brown algae	*Sargassum muticum*
MR-19	Coastal sponge	Porifera; Family Suberitidae
MR-20	Brown algae	*Desmarestia aculeata*
MR-21	Red algae	*Polysiphonia stricta*
MR-22	Coastal sponge	*Polymastia boletiformis*
MR-23	Brown algae	*Sargassum muticum*
MR-24	Coastal sponge	*Suberites ficus*
MR-25	Coastal sponge	*Dysidea fragilis*

MR-26(1)	Tunicate	Ascidian; Family Styelidae
MR-27	Coastal sponge	*Haliclona simulans*
MR-28(2)	Tunicate	Ascidian; Family Styelidae
MR-29	Coastal sponge	*Haliclona indistincta*
MR-30	Brown algae	*Cystoseira baccata*
MR-31	Brown algae	*Desmarestia viridis*
MR-32	Brown algae	*Desmarestia ligulata*
MR-33	Coral	Soft coral
MR-34	Hydrozoan	Hydrozoan
MR-35	Coastal sponge	Sea fan; Paramuricea
MR-36	Sea star	Sea star
MR-37	Brittle star	N/A
MR-38	Brown algae	*Cystoseira foeniculacea*
MR-39	Brown algae	*Cystoseira nodicaulis*
MR-40	Deep sea sponge	*Lissodendoryx diversichela*
MR-41	Sea cucumber	N/A
MR-42	Sea fan	N/A
	Ethanol dissolved extracts	
87	Star fish	N/A
79	Sea fan	N/A
35	Deep sea sponge	*Haliclona simulans*
199	Soft coral	Soft coral
146	Sea star	Sea star
1448	Sea star	Cushion star fish
1544	Sea star	N/A
1095	Sea star	Sea star
1133	Sea pen	Sea pen
1359	Sea star	Cushion sea star
1083	Sea fan	*Phanophathes sp.*
12	Red algae	*Corallina elongata*
279	Algae (griffithsia)	*Griffithsia corallinoides*
B1295	Sea star	Cushion sea star
B1360	Sea fan	*Pentaculacea*

B1693	Deep sea sponge	*Haliclona indistincta*
614	Red algae	*Ceramium secundatum*
324	Brown algae	*Bifurcaria bifurcata*
294	Red algae	*Boergeseniella fructiculosa*
333	Red algae	*Plocamium cartilagineum*
625	Red algae	*Osmundea sp.*
621	Brown algae	Ectocarpales (Puncatria)
632	Sponge	*Cladostephus spongiosus*
B13568	Coral	N/A
B787	Deep sea sponge	*Haliclona indistincta*

Appendix 7 – powder extract cytotoxicity

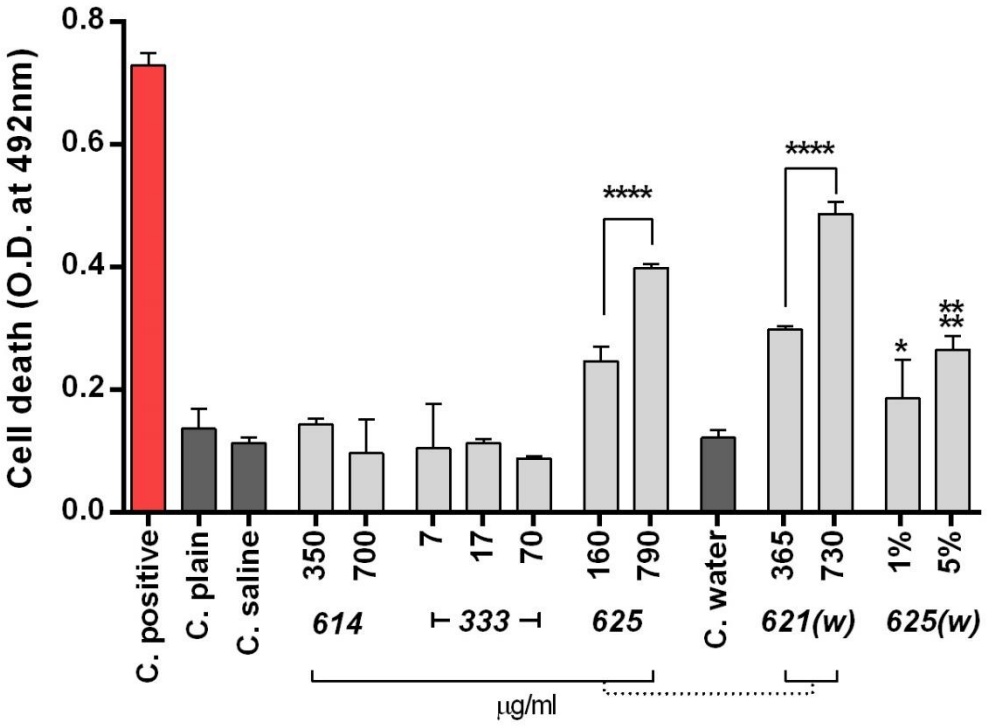

Figure 8.2: cytotoxicity results (LDH assay) for hBMSCs (donor:002, passage:5). Treatments include powder extracts dissolved in saline (614, 333 and 625) or water [(621(w) and 625 (w)] solution at a range of concentrations. Cells were given a 24 h attachment period, 24 h with treatment and then supernatant was collected for testing. Results are presented as the mean+/- standard deviation (n=3). C+ve (red) shows the absorbance values for 100% cell death. All values are corrected for background absorbance by subtracting appropriate negative control values, run on the same plate. Kit positive control confirmed the assay worked on each plate as expected. Controls included complete media (C. plain) and complete media with saline solution (1-10%) or water (1/5%). Statistical significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ between the stated treatment and relevant control.

Appendix 8 – operculum system mortality tables

Note: in chapter 5 results for similar taxa or water/saline treatments of the same extract were grouped together. However, this does not reflect the order in which extracts were tested in each experiment and therefore how they are presented here.

Table 8.3: mortality table showing the death of larval zebrafish (tested in the operculum area system) during the three-day exposure period to extracts. Mortality is shown at 24, 48 and 72 hours, as well any losses on the picture day (due to damage during placement for imaging) or exclusions of obvious outliers (underlined). Total losses as a % of population are also shown. Control treatments are included, as well as varying concentrations of extracts 632 water (*C. spongiosus*) and 1358 water (*P. squamatus*). For all treatments n=15. See figures 5.15 and 5.16.

Treatment	Treatment	Mortality (hours with treatment)			Losses/exculsions	Total losses
(Water)	level	24	48	72	Pic day	% of population
632	400 µg/ml			4		26.7
632	200 µg/ml	1				6.7
1358	160 µg/ml				1	6.7
1358	1.3 µg/ml				1	6.7
1358	0.65 µg/ml				1	6.7
C (negative)	10% dH2O	No mortality				
C (ethanol)	0.1%				1	6.7
C (vitamin D)	10 pg/ml				1	6.7

Table 8.4: mortality table showing the death of larval zebrafish (tested in the operculum area system) during the three-day exposure period to extracts. Mortality is shown at 24, 48 and 72 hours, as well any losses on the picture day (due to damage during placement for imaging) or exclusions of obvious outliers (underlined). Total losses as a % of population are also shown. Control treatments are included, as well as varying concentrations of extracts 840 water (*S. muticum*) and 294 water (*B. fruticulosa*). For all treatments n=10. See figures 5.14 and 5.11.

Treatment	Treatment	Mortality (hours with treatment)			Losses/exculsions	Total losses
(water)	level	24	48	72	Pic day	% of population
840 (water)	580 µg/ml	10	(total mortality)			100
840 (water)	290 µg/ml	10	(total mortality)			100
840 (water)	58 µg/ml	6				60
294 (water)	5%				1	10
294 (water)	0.04%				1	10
C (negative)	10% dH2O				1	10
C (ethanol)	0.1%	No mortality				
C (vitamin D)	10 pg/ml			1	1	20

Table 8.5: mortality table showing the death of larval zebrafish (tested in the operculum area system) during the three-day exposure period to extracts. Mortality is shown at 24, 48 and 72 hours, as well any losses on the picture day (due to damage during placement for imaging) or exclusions of obvious outliers (underlined). Total losses as a % of population are also shown. Control treatments are included, as well as varying concentrations of extracts 614 water (*C. secundatum*) and 333 water (*P. lyngbyanum*). For all treatments n=15. See figures 5.9 and 5.6.

Treatment	**Treatment**	**Mortality (hours with treatment)**			**Losses/exculsions**	**Total losses**
(water)	**level**	**24**	**48**	**72**	**Pic day**	**% of population**
614	340 µg/ml				1	6.7
614	140 µg/ml				2	13.3
614	1.4 µg/ml				1	6.7
333	1%				1	6.7
333	0.2%				2	13.3
C (negative)	10% dH_2O	No mortality				
C (ethanol)	0.1%				1	6.7
C (vitamin D)	10 pg/ml	No mortality				

Table 8.6: mortality table showing the death of larval zebrafish (tested in the operculum area system) during the three-day exposure period to extracts. Mortality is shown at 24, 48 and 72 hours, as well any losses on the picture day (due to damage during placement for imaging) or exclusions of obvious outliers (underlined). Total losses as a % of population are also shown. Control treatments are included, as well as varying concentrations of extracts 840 saline (*S. muticum*), 1358 saline (*P. squamatus*), 632 saline (*C. spongiosus*). For all treatments n=10. See figures 5.14, 5.16 and 5.15.

Treatment	**Treatment**	**Mortality (hours with treatment)**			**Losses/exculsions**	**Total losses**
(saline)	**level**	**24**	**48**	**72**	**Pic day**	**% of population**
840	760 µg/ml	7	3			100
840	380 µg/ml	2	6	1		90
1358	830 µg/ml		1			10
1358	166 µg/ml		2			20
1358	33 µg/ml				1	10
632	640 µg/ml				1	10
632	320 µg/ml				1	10
C (negative)	10% saline	No mortality				
C (ethanol)	0.1%	No mortality				
C (vitamin D)	10 pg/ml			1		10

Table 8.7: mortality table showing the death of larval zebrafish (tested in the operculum area system) during the three-day exposure period to extracts. Mortality is shown at 24, 48 and 72 hours, as well any losses on the picture day (due to damage during placement for imaging) or exclusions of obvious outliers (underlined). Total losses as a % of population are also shown. Control treatments are included, as well as varying concentrations of extracts 615 water (*C. pallidum*) and 621 water (*Punctaria sp.*). For all treatments n=15. See figures 5.8 and 5.13.

Treatment	Treatment	Mortality (hours with treatment)			Losses/exculsions	Total losses
(water)	level	24	48	72	Pic day	% of population
615	1500 µg/ml	1			1	13.3
615	750 µg/ml	1				6.7
615	150 µg/ml			11		73.3
615	30 µg/ml		1	1	1	20.0
621	N/A	No mortality in any treatment				
C (negative)	10% dH2O		1	2	1	26.7
C (ethanol)	0.2%	No mortality				
C (vitamin D)	20 pg/ml				1	6.7

Table 8.8: mortality table showing the death of larval zebrafish (tested in the operculum area system) during the three-day exposure period to extracts. Mortality is shown at 24, 48 and 72 hours, as well any losses on the picture day (due to damage during placement for imaging) or exclusions of obvious outliers (underlined). Total losses as a % of population are also shown. Control treatments are included, as well as varying concentrations of extracts 333 saline (*P. lyngbyanum*) and 614 saline (*C. secundatum*). For all treatments n=15. See figures 5.9 and 5.6.

Treatment	Treatment	Mortality (hours with treatment)			Losses/exculsions	Total losses
(saline)	level	24	48	72	Pic day	% of population
333	820 µg/ml			1	1	13.3
333	410 µg/ml			1	1	13.3
614	N/A	No mortality in any treatment				0
C (negative)	10% saline	No mortality				0
C (ethanol)	0.15%	No mortality				0
C (vitamin D)	15 pg/ml				1	6.7

Table 8.9: mortality table showing the death of larval zebrafish (tested in the operculum area system) during the three-day exposure period to extracts. Mortality is shown at 24, 48 and 72 hours, as well any losses on the picture day (due to damage during placement for imaging) or exclusions of obvious outliers (underlined). Total losses as a % of population are also shown. Control treatments are included, as well as varying concentrations of UK and Ireland-derived extracts 334 saline (*P. cartilagineum*) and 614 saline (*C. secundatum*). For all treatments n=15. See figures 5.10 and 5.7.

Treatment	Treatment	Mortality (hours with treatment)			Losses/exculsions	Total losses
(saline)	level	24	48	72	Pic day	% of population
334 UK	22 µg/ml				1	6.7
614 Ire	525 µg/ml			1		6.7
614 Ire	105 µg/ml				1	6.7
C (negative)	5% saline			1		6.7
C (ethanol)	0.15%	No mortality				0
C (vitamin D)	15 pg/ml	No mortality				0

Table 8.10: mortality table showing the death of larval zebrafish (tested in the operculum area system) during the three-day exposure period to extracts. Mortality is shown at 24, 48 and 72 hours, as well any losses on the picture day (due to damage during placement for imaging) or exclusions of obvious outliers (underlined). Total losses as a % of population are also shown. Control treatments are included, as well as varying concentrations of extract 294 saline (*B. fruticulosa*) and 615 saline (*C. pallidum*). For all treatments n=15. See figures 5.11 and 5.8.

Treatment	Treatment	Mortality (hours with treatment)			Losses/exculsions	Total losses
(saline)	level	24	48	72	Pic day	% of population
294	1400 µg/ml	7	2	6		100
294	700 µg/ml	6				40
615	1600 µg/ml	2	13			100.0
615	800 µg/ml			1	1	13.3
C (negative)	10% saline	No mortality				0
C (ethanol)	0.15%	No mortality				0
C (vitamin D)	15 pg/ml	No mortality				0

Table 8.11 mortality table showing the death of larval zebrafish (tested in the operculum area system) during the three-day exposure period to extracts. Mortality is shown at 24, 48 and 72 hours, as well any losses on the picture day (due to damage during placement for imaging) or exclusions of obvious outliers (underlined). Total losses as a % of population are also shown. Control treatments are included, as well as varying concentrations of extract 625 in either saline solution or water. For all treatments n=15. See figure 5.12.

Treatment	Treatment	Mortality (hours with treatment)			Losses/exculsions	Total losses
	level	24	48	72	Pic day	% of population
625 saline	1600 µg/ml		11	1		80
625 saline	800 µg/ml		9			60
625 saline	32 µg/ml				1	6.7
625 water	1200 µg/ml		6	1	1	53.3
625 water	600 µg/ml		1	1		13.3
625 water	120 µg/ml		1			6.7
C (negative)	10% saline	No mortality				
C (ethanol)	0.15%	No mortality				
C (vitamin D)	15 pg/ml				1	6.7

Appendix 9 – list of samples analysed via GC-FID

Table 8.12: full list of samples analysed via GC-FID. Included is the order they were tested in (analysis no.), the species/genus name for each extract along with their extract ID and whether the sample was dissolved in water or saline solution. Concentration has not been included as extracts were tested in their pure form and concentration was only determined for extracts in solution (i.e. culture media or zebrafish water). However, for each extract concentration is likely to be in the range of 10-20 mg/ml. In addition to extracts a standard FAME mix was included for reference.

Analysis no.	Species/genus	Extract ID	Solvent
1	*P. squamatus*	1358	water
2	*P. squamatus*	1358	saline
3	*P. cartilagineum*	334 Ire	saline
4	*P. cartilagineum*	334 UK	water
5	*P. cartilagineum*	334 UK	saline
6	*P. cartilagineum*	334 Ire	water
7	*P. lyngbyanum*	333	saline
8	*C. spongiosus*	632	water
9	*C. spongiosus*	632	saline
10	*C. secundatum*	614	saline
11	*C. secundatum*	614	water
12	*C. secundatum*	614 UK	water
13	*C. secundatum*	614 Ire	water
14	*C. secundatum*	614 UK	saline
15	*C. secundatum*	614 Ire	saline
16	*B. fruticulosa*	294	saline
17	*S. muticum*	840	saline
18	*S. muticum*	840	water
19	*Osmundea sp.*	625	saline
20	*Punctaria sp.*	621	water
21	*C. pallidum*	615	water
22	*C. pallidum*	615	saline

Appendix 10 – samples analysed via mass spec

Table 8.13: samples analysed via mass spec in positive ionisation mode. Included is the order they were tested in (analysis no.), the species/genus name for each extract along with their extract ID and whether the sample was dissolved in water or saline solution.

Analysis no.	Species/genus	Extract ID	Solvent
6	*P. cartilagineum*	334 Ire	Saline
7	*C. secundatum*	614 Ire	Saline
8	*C. secundatum*	614 UK	Saline
9	*C. pallidum*	615	water

Appendix 11 – example GC-FID analysis

As GC-FID analysis is thought to have only shown impurities, rather than identifiable fatty acids, only one example analysis (opposed to all 22) has been included.

```
Acq. Operator    : DANIEL                          Seq. Line :   2
Acq. Instrument  : GC 2                             Location : Vial 21
Injection Date   : 26-Jul-17, 18:10:06                   Inj :   1
                                                  Inj Volume : 1 µl
Acq. Method      : C:\Chem32\1\DATA\DAN\DEF_GC 2017-07-26 17-37-31\FAME HT.M
Last changed     : 25/07/2017 04:00:40 PM by DANIEL
Analysis Method  : C:\CHEM32\1\DATA\DAN\DEF_GC 2017-07-26 17-37-31\FAME HT.M
Last changed     : 27/07/2017 09:31:47 AM by YH SONG
                   (modified after loading)
======================================================================
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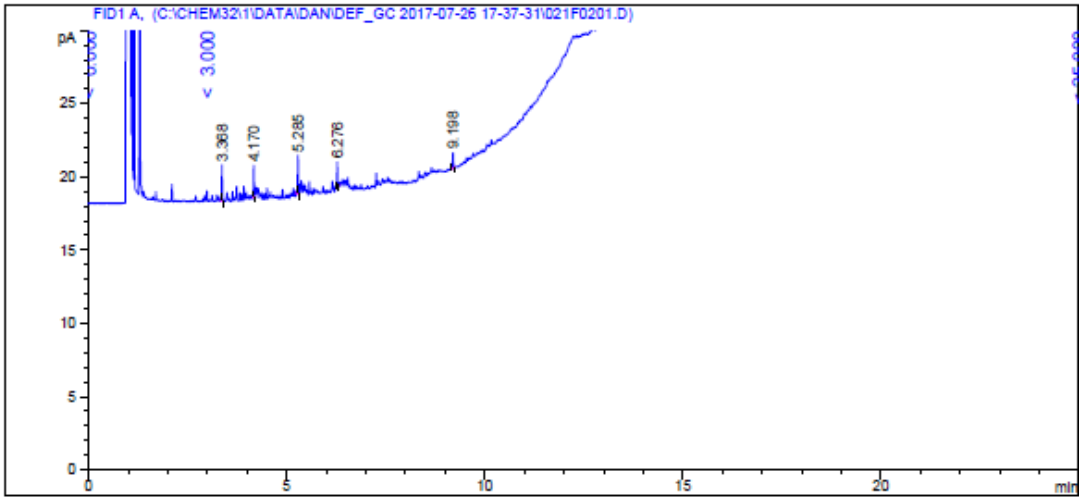


```
======================================================================
                        Area Percent Report
======================================================================

Sorted By             :      Signal
Calib. Data Modified  :      13 December 2013 12:04:33 PM
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: FID1 A,

Peak RetTime  Type   Width     Area      Area     Name
  #   [min]           [min]   [pA*s]       %
----|-------|------|-------|----------|--------|--------------------
   1   3.368 BB      0.0154    2.35569 20.94596 ?
   2   4.170 BB      0.0199    2.85564 25.39131 ?
   3   5.285 BB      0.0176    2.83958 25.24856 ?
   4   6.276 BB      0.0171    1.72655 15.35183 ?
   5   9.198 BB      0.0222    1.46906 13.06235 ?
   6  12.179         0.0000    0.00000  0.00000 1-methylnaphthalene

Totals :                      11.24651
```

Figure 8.3: example GC-FID analysis for *P. squamatus* (1358) in water (analysis/readout 1 of 22).

Appendix 12 – mass spectrometry analysis

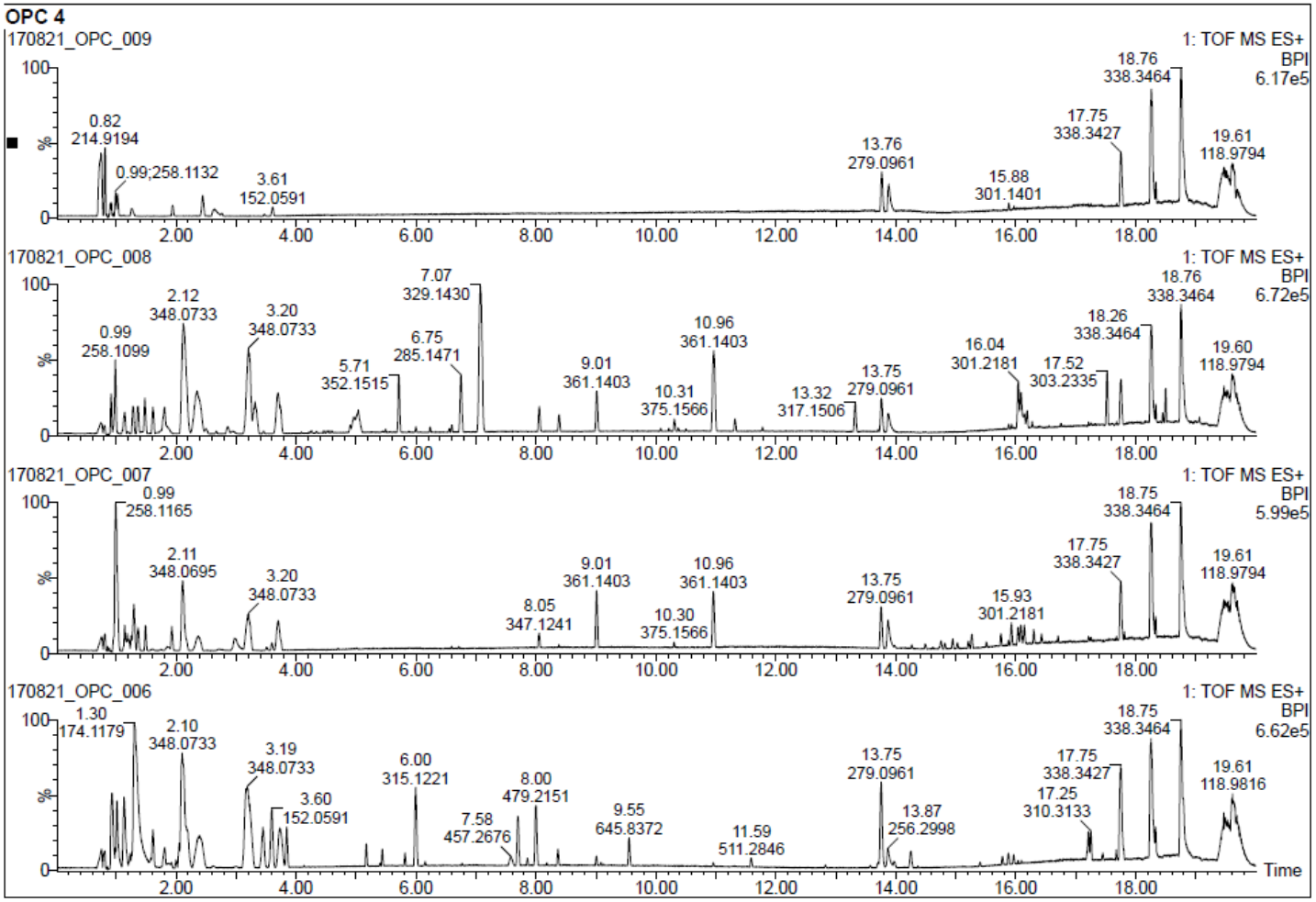

Figure 8.4: mass-spectrometry (positive ionisation mode) analysis for 4 samples: see table 7.12.